Single-cell immune profiling of atherosclerosis: from omics to therapeutics

Depuydt, M.A.C.

Chapter 2

The application of single-cell transcriptomics in healthy and diseased vasculature

Manuscript in preparation

Marie A.C. Depuydt[1]*, Carlijn M. Lems[1]*, Bram Slütter[1], Ilze Bot[1]

1. Division of BioTherapeutics, LACDR, Leiden University

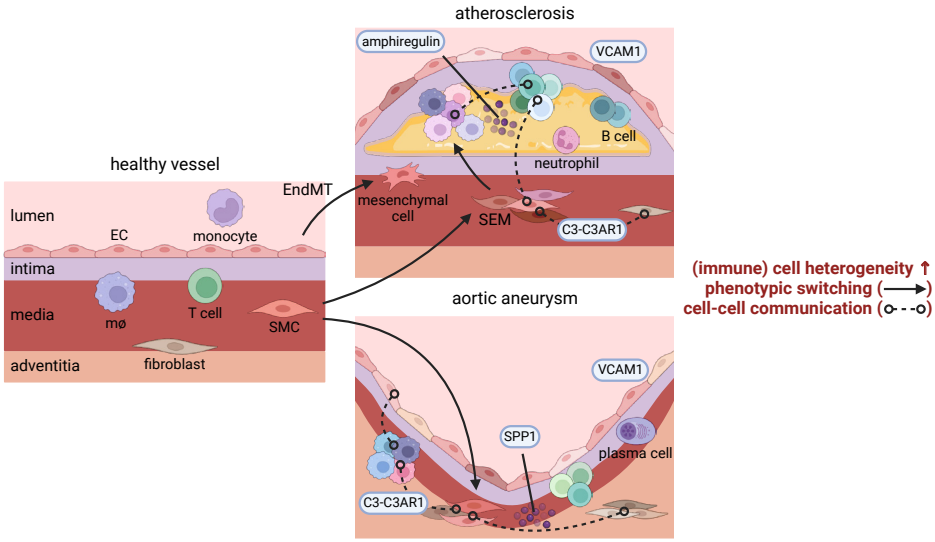
*These authors contributed equally

Abstract

Despite therapeutic advances, cardiovascular complications such as atherosclerosis and aortic aneurysm (AA) continue to be the primary cause of death worldwide. This emphasizes the urgent need to elucidate the complex biological mechanisms that underpin vascular disease formation and progression. Recent advances in single-cell RNA sequencing have provided new insights into the heterogeneity of cell populations, cellular transitions into pathological phenotypes, and cell-cell communication within the diseased vascular wall.

In this review, we summarize these findings and discuss the future perspectives of single-cell transcriptomics applications in studies of atherosclerosis and AA. Single-cell RNA sequencing has revealed the cellular landscape of atherosclerotic plaques and AA tissues to be much more diverse than previously believed, in particular with regard to immune cells. It has also characterized the enormous potential of vascular cells, in particular smooth muscle cells, for phenotypic switching and how this contributes to disease. Additionally, single-cell RNA sequencing has contributed to elucidating the complex cellular interaction network that governs vascular disease, thereby discovering candidate signaling pathways and ligand-receptor pairs for drug discovery. Lastly, this technique has uncovered both striking similarities and distinct differences between atherosclerosis and AA.

Single-cell transcriptomics has been of immense value in deepening our understanding of atherosclerosis and AA pathogenesis, thereby paving the way for the development of novel therapies. The ever-increasing amount of available data offers a unique opportunity for integrated and cross-species analyses to unify cell population nomenclature. Directions for future research include combining single-cell RNA sequencing with various single-cell omics techniques and outcomes of genome-wide association studies.



Graphical abstract. Summary of the cellular content, cellular plasticity and the main cellular crosstalk pathways in the healthy and diseased vasculature as described by single-cell transcriptomics. C3, complement component 3; C3AR1, complement C3a receptor 1; EC, endothelial cell; EndMT, endothelial-to-mesenchymal transition; mø, macrophage; SEM, stem cell, endothelial cell, monocyte; SPP1, secreted phosphoprotein 1; VCAM1, vascular cell adhesion molecule 1. The figure was created in BioRender.com.

Introduction

Cardiovascular disease is the leading cause of global mortality and its prevalence as well as its number of associated deaths are continuously increasing.[1] Common cardiovascular complications include atherosclerosis and aortic aneurysm (AA)[2]. Atherosclerosis (reviewed in [3]) is the primary underlying pathology of cardiovascular syndromes such as myocardial infarction or stroke worldwide and is characterized by chronic inflammation and the formation of atheromatous plaques in the vascular wall. Such plaques are typically covered with a thin fibrous cap and are rich in lipids and locally infiltrating inflammatory cells that promote the degradation of extracellular matrix (ECM) molecules such as collagen. Advanced atherosclerotic lesions can obstruct blood flow, either by gradually infringing on the arterial lumen or by provoking thrombus formation following plaque rupture or erosion, both leading to ischemia. This may trigger acute cardiovascular syndromes such as myocardial infarction or stroke. Established risk factors for atherosclerosis include hypercholesterolemia, diabetes, smoking, and hypertension. Genetic conditions such as familial hypercholesterolemia may also increase the risk of atherosclerosis.

After atherosclerosis, AA (reviewed in [4,5]), defined as a localized dilatation of the aorta, is the most common disease affecting the aorta. It results from structural changes in the aortic wall, including thinning of the outer layers due to the progressive loss of vascular smooth muscle cells (SMCs) and degradation of the ECM. This increases the susceptibility of the aorta to rupture, causing a potentially life-threatening hemorrhage. AAs can be subdivided into two groups based on anatomic location, namely thoracic aortic aneurysm (TAA) and abdominal aortic aneurysm (AAA). Important risk factors implicated causally in AAA formation are smoking, male sex, and the presence of atherosclerosis. Conversely, genetic predisposition plays a more prominent role in TAA, with conditions such as Marfan syndrome (MFS) comprising important risk factors.

For many years, researchers have sought to elucidate the various mechanisms that underpin vascular pathology development and progression. In the early years, knowledge was largely derived from pathology and histology research, which provided valuable information on healthy and diseased vascular tissue at the level of individual markers. This was then followed by the emergence of more modern techniques such as conventional bulk transcriptomics, which enabled the simultaneous characterization of entire gene expression profiles. Although this method has considerably increased our knowledge of differential gene expression in healthy versus diseased vascular systems, an important limitation is that it measures average gene expression across cells and hence does not allow for profiling populations on a single-cell level. A single-

cell resolution is required to adequately capture the large cellular heterogeneity in the arterial wall. Single-cell RNA sequencing (scRNA-seq) was first reported in 2009 and has quickly become a key technique in the domain of vascular pathology [6] Its main advantages lie in the identification of rare or *de novo* cell subpopulations[7], the ability to trace the lineage of cells[8], and the elucidation of cellular crosstalk.[9]

There is a rapidly growing body of literature on scRNA-seq efforts in atherosclerosis and AA. Additionally, exploration and re-analysis of the various publicly available datasets are facilitated by web portals such as PlaqView.[10,11] What remains unclear, however, is precisely what contribution scRNA-seq has made to enhancing our understanding of vascular diseases at a cellular level, and how this technique can be optimally employed in the future. This review aims to assess the value of scRNA-seq in vascular disease research by summarizing findings in healthy vasculature and the vascular pathologies atherosclerosis and aortic aneurysm, and by discussing future opportunities for its application in this field. This work offers a fresh perspective on the worth and untapped potential of a fairly new experimental approach in an increasingly important field of research and may guide future research efforts. First, we provide an overview of the current knowledge owing to scRNA-seq research in mice and humans on the cellular composition of normal and diseased vasculature, lineage-tracing experiments, and intercellular communication in the vascular wall. We then draw together these various findings to critically reflect on the added value of scRNA-seq in vascular disease research up until now. Finally, we propose several promising future research directions, such as spatial transcriptomics and single-cell multi-omics approaches.

Current knowledge of healthy and diseased vasculature: insights from scRNA-seq

Cellular composition of vasculature

Healthy vasculature comprises a wide variety of cell types, including endothelial cells (ECs), vascular SMCs, immune cells, and fibroblasts, all of which in turn consist of several subpopulations. ECs thinly line the interior surface of blood vessels, thereby functioning as a barrier between the circulation and tissues. Additionally, they control vascular relaxation and contraction by releasing vasoactive substances, and they regulate blood coagulation. Vascular SMCs are located in the media of the vascular wall. Here, they provide structural support and are primarily responsible for regulating arterial tone to control blood pressure and blood flow. Vascular SMCs exhibit two distinct phenotypes, namely a quiescent, contractile phenotype and a proliferative, undifferentiated, synthetic phenotype. Of note, based on a review of scRNA-seq data,

Yap *et al.* classified four different SMC phenotypes besides a contractile phenotype and a central dedifferentiated phenotype.[12] Hence, the authors argue that the classification of vascular SMCs should be expanded beyond the contractile and synthetic profiles. Some immune cell subsets, including T cells, B cells, and macrophages, 'patrol' the adventitia of healthy arteries[13], albeit in limited numbers compared to ECs and SMCs. In contrast, other immune cell subsets are sparsely found in normal arteries, such as neutrophils. Immune cells play a pivotal role in maintaining vascular health and are recruited from the vascular adventitia and lumen in response to inflammatory signals. Lastly, fibroblasts contribute to the structural framework of blood vessels through the production of ECM and the construction of fiber networks.

Single-cell transcriptomics has not only confirmed our previous knowledge of healthy vasculature but also underlies major recent advances in vascular biology (reviewed in [14]). Above all, it has enabled the identification and in-depth characterization of cellular subpopulations[15–19], including ECs[20–23], SMCs[24–26], and macrophages[27,28]. For instance, scRNA-seq of healthy murine aortas recently revealed 10 different cell clusters representing the four aforementioned primary vascular cell types.[20] Surprisingly, not SMCs but ECs showed the highest cellular heterogeneity. Kalluri *et al.* identified three functionally distinct EC subpopulations with concomitant gene expression profiles suggesting specialization in either angiogenesis, ECM production, and lipoprotein handling (ECs 1 and 2) or lymphatic function (EC 3).[20] Transcriptional markers that differentiate these three populations include, respectively, canonical EC markers (*Vcam1*), genes related to angiogenesis (*Flt1*) or lipid transport (*Cd36*, *Lpl*), and markers of lymphatic identity (*Lyve1*). With regard to vascular SMCs, Dobnikar *et al.* detected seven subpopulations in healthy mouse vessels, including a rare *Sca1*$^{+}$ population. [25] This *Sca1*$^{+}$ SMC subset showed a progressive downregulation of contractile genes (*Myh11*, *Actn4*) and upregulation of genes involved in ECM synthesis (*Mgp*, *Col8a1*, *Spp1*), migration (*Pak3*, *Igf1*, *Igfbp5*), and proliferation. Moreover, this study revealed that *Sca1* upregulation is a hallmark of SMC phenotypic switching. Furthermore, the emergence of single-cell transcriptomic atlases, such as the Tabula Sapiens[29], has uncovered tissue-specific features of cell types across human organs (reviewed in [30]), including in their blood vessels. This is of importance for understanding differences between vascular diseases affecting different tissues, e.g., the aorta and the heart.

Atherosclerosis

The cellular landscape of atherosclerotic plaques has been constructed the most extensively using single-cell transcriptomics. An overview of scRNA-seq experiments conducted in atherosclerosis research is presented in **Table 1**. In the next paragraphs, we will discuss the individual cell populations, including ECs, SMCs, and various immune cells.

Endothelial cells

Endothelial dysfunction is one of the main drivers of atherosclerosis initiation.[53] Wirka *et al.* identified two EC clusters, defined by genes such as *Pecam1*, *Egfl7*, and *Esam* (EC 1), and *Lrg1*, *Mmm1*, and *Ecscr* (EC 2), in mouse atherosclerotic aortas.[33] Together, these clusters mapped to a single EC cluster in diseased human coronary arteries. Conversely, several scRNA-seq studies report the presence of two major EC clusters in human carotid atherosclerotic plaques.[35,50,51] These populations were enriched in *COL4A1/2*[50,51], *SPARCL1*[50], and *PLVAP* (EC 1), and *MPZL2*, *SULF1*, and *VWF* (EC 2). Depuydt *et al.* additionally performed further subclustering to reveal four distinct EC subpopulations.[50] Three of these subpopulations represented activated endothelium characterized by angiogenic capacity (*PRCP*), cell adhesion, and the facilitation of leukocyte extravasation (*VCAM1*), thereby actively stimulating lesion inflammation. Furthermore, one EC subset displayed clear signs of endothelial-to-mesenchymal transition (EndMT) (*ACTA2*, *MYH11*)[50], a phenomenon that we discuss in more detail in the lineage tracing section below.[54] Another study showed that inflammatory ECs (*IL6*, *ACKR1*, *HLA-DQA1*) are located in the proximal adjacent region of diseased human carotid arteries[52], whereas ECs in the atherosclerotic core appear to be involved in intimal repair (*ITLN*, *DKK2*) and ECM modulation (*FN1*).

Smooth muscle cells

Similarly, single-cell transcriptomics has been applied to describe yet unknown layers of SMC phenotypic heterogeneity in atherosclerosis. The majority of findings concern the phenotypic switching or modulation of SMCs which is a hallmark of atherosclerosis.[55,56] We provide an in-depth discussion of SMC modulation-related findings generated through trajectory analysis the lineage tracing section below. An early scRNA-seq finding was that an equivalent of the abovementioned *Sca1*$^{+}$ SMC population found in healthy mouse vessels is present in murine atherosclerotic plaques [25]. This was confirmed by Wang *et al.*[39], who additionally showed that this highly de-differentiated *Sca1*$^{+}$ subset clonally expands during mouse atherogenesis. Aside from downregulating classic SMC markers, these SMCs were enriched in genes associated with inflammation, innate immunity, and the classical complement cascade, particularly *C3*. Although a gene signature resembling murine *Sca1*$^{+}$ SMCs was discovered in bulk RNA-seq data of human atherosclerosis[39], a homolog of *Sca1* is absent in humans.[57] In human carotid plaques, histone variant H2A.Z is pivotal for maintaining vascular SMC identity [48], as indicated by a downregulation of SMC marker genes (*CALD1*, *ACTA2*) and an upregulation of the proliferation-promoting gene *SMARCA4* in *H2AFZ*^{low} SMCs.

Table 1. Single-cell transcriptomics studies in atherosclerotic tissue.

Sex	Genotype	Tissue
Mouse		
Female	*ApoE*$^{-/-}$	Aorta CD45^{+}
Unknown	*Ldr*$^{-/-}$	Aorta CD45^{+}
Male	*LdlR*$^{-/-}$	Aorta CD45^{+}
Male	*ApoE*$^{-/-}$	Aortic foam cells
Male	C57BL/6	Aorta
Male	*ApoE*$^{-/-}$; *Myh11-CreERt2*; *Confetti*	Aorta
Male	*LdlR*$^{-/-}$	Aorta CD45^{+}
Female	*ApoE*$^{-/-}$	Aorta CD45^{+}
Male	C57BL/6J, *ApoE*$^{-/-}$	Aortic adventitia
Male	*ApoE*$^{-/-}$; *Tcf21*$^{+/+}$; *Tg*$^{Myh11\text{-}CreERT2}$; *ROSAtdT*$^{f/+}$, *ApoE*$^{-/-}$; *Tcf21*$^{\Delta SMC/\Delta SMC}$; *Tg*$^{Myh11\text{-}CreERT2}$; *ROSAtdT*$^{f/+}$	Aortic root/ascending aorta
Unknown	*Cx3cr1*$^{CreERT2\text{-}IRES\text{-}YFP/+}$; *Rosa26*$^{fl\text{-}tdTomato/+}$; *Ldr*$^{-/-}$ (AAV-mPCSK9)	Aortic arch
Male	*ROSA26*$^{ZsGreen1/+}$; *Ldr*$^{-/-}$; *Myh11-CreER*^{T2}, *ROSA26*$^{ZsGreen1/+}$; *ApoE*$^{-/-}$; *Myh11-CreER*^{T2}	Aorta
Unknown	C57BL/6	Aorta CD45^{+}
Unknown	*CX3CR1*$^{CreER/+}$; *Rosa26-LSL-Tomato*; *Ldr*$^{-/-}$	Aorta CD45^{+}
Male	*Myh11-Cre*^{ERT2}; *Rosa-eYFP*; *ApoE*$^{-/-}$	BCA
Male	*Myh11-Cre*^{ERT2}; *Rosa-eYFP*; *ApoE*$^{-/-}$, *Cdh5-Cre*^{ERT2}; *Rosa-eYFP*; *ApoE*$^{-/-}$, *Myh11-Cre*^{ERT2}; *Rosa-eYFP*; *ApoE*$^{-/-}$; *SMCKlf4*$^{WT/WT}$, *Myh11-Cre*^{ERT2}; *Rosa-eYFP*; *ApoE*$^{-/-}$; *SMCKlf4*$^{\Delta/\Delta}$	BCA
Male and female	*Myh11-Dre*^{ERT2}; *Lgals3-Cre*; *Rosa-tdTomato-eGFP*; *ApoE*$^{-/-}$	BCA

	Exposure	Cell type focus	References
	WD 12 weeks	Immune cells	Winkels *et al.*, 2018 [31]
	CHD 12 weeks		
	HCD 12 weeks		
	CHD 12 weeks		
	WD 12 weeks	Macrophages	Kim *et al.*, 2018 [32]
	WD 27 weeks		
	-	SMCs	Dobnikar *et al.*, 2018 [25]
	WD 14/18 weeks		
	HFD 11 weeks	Macrophages	Cochain *et al.*, 2018 [28]
	CHD 11 weeks		
	HFD 20 weeks		
	HFD 12 weeks		
	CHD	Immune cells, non-immune cells	Gu *et al.*, 2019 [15†,*]
	HFD 0/8/16 weeks	SMCs	Wirka *et al.*, 2019 [33]
	WD 20 weeks	Macrophages	Lin *et al.*, 2019 [34†]
	WD 18 weeks +		
	CHD 2 weeks + ApoB-ASO		
	WD 0/8/16/26 weeks	SMCs	Pan *et al.*, 2020 [35]
	-	Macrophages	Williams *et al.*, 2020 [36]
	HFD 3 weeks		
	-	SMCs	Alencar *et al.*, 2020 [37]
	WD 18 weeks		
	WD 18 weeks		

Table 1. Continued.

Sex	Genotype	Tissue
Male	*Ldlr*$^{-/-}$	Aorta CD45^{+}
Male	*Myh11-Cre*^{ERT2}; *Rosa26*$^{tdTomato/tdTomato}$; *ApoE*$^{-/-}$	Aortic arch
Male	*Tg*$^{Myh11\text{-}CreERT2}$; *ROSA*$^{tdT/tdT}$; *ApoE*$^{-/-}$, *Tg*$^{Myh11\text{-}CreERT2}$; *ROSA*$^{tdT/tdT}$; *ApoE*$^{-/-}$; *Ahr* $^{\Delta SMC/\Delta SMC}$	Aortic root
Female	*ApoE*$^{-/-}$	Aorta CD45^{+}
Male and female	C57BL6/J (AAV-mPCSK9)	Aortic arch CD45^{+}
Male	*Ntn1*$^{fl/fl}$*Cx3cr1*$^{CreERT2+}$, *Ntn1*$^{fl/fl}$*Cx3cr1*^{WT} (AAV-mPCSK9)	Aortic arch CD45^{+}
Male	*Ldlr*$^{-/-}$	Aortic arch CD45^{+}
Male	*ApoE*$^{-/-}$	Aorta CD45^{+}
Male	*ApoE*$^{-/-}$	Aorta CD45^{-}
Male	*Perk*$^{SMC+/+}$ (AAV-mPCSK9), *Perk*$^{SMC-/-}$ (AAV-mPCSK9)	Aortic root to distal aortic arch
Human		
Unknown		Carotid plaque
Male and female		Coronary artery
Male and female		Carotid plaque CD45^{+}, blood CD45^{+}
Male and female		Carotid plaque
Male and female		Carotid plaque
Male and female		Carotid plaque
Male and female		Carotid plaque
Unknown		Carotid plaque

AAV-mPCSK9, adeno-associated virus vector encoding mouse proprotein convertase subtilisin/kexin type 9; ApoB-ASO, antisense oligonucleotide to apolipoprotein B; BCA, brachiocephalic artery; CHD, chow diet; EC, endothelial cell; HCD, high-cholesterol diet; HFD, high-fat diet;

Exposure	Cell type focus	References
HFD 10-11 weeks CHD 10-11 weeks	Neutrophils	Vafadarnejad *et al.*, 2020 [38]
HFD 18 weeks	SMCs	Wang *et al.*, 2020 [39]
HFD 16 weeks	SMCs	Kim *et al.*, 2020 [40]
WD 12 weeks CHD 12 weeks	T cells	Wolf *et al.*, 2020 [41]
WD 20 weeks WD 20 weeks + CHD 3 weeks + ApoB-ASO + IgG or anti-CD25	T cells	Sharma *et al.*, 2020 [42]
WD 20 weeks WD 20 weeks + CHD 4 weeks	Monocytes/macrophages	Schlegel *et al.*, 2021 [43†]
WD 14 weeks + CHD 4 weeks + control anti-miR or anti-miR-33	Immune cells	Afonso *et al.*, 2021 [44]
NCD 16 weeks HCD 11 weeks	Macrophages	Burger *et al.*, 2022 [45]
NCD 16 weeks HCD 11 weeks	SMCs	Brandt *et al.*, 2022 [46]
HFD 12 weeks	SMCs	Chattopadhyay *et al.*, 2022 [47†]
	SMCs	Yao *et al.*, 2018 [48]
	SMCs	Wirka *et al.*, 2019 [33]
	Immune cells	Fernandez *et al.*, 2019 [49*]
	ECs, SMCs, immune cells	Depuydt *et al.*, 2020 [50*]
	SMCs	Pan *et al.*, 2020 [35]
	SMCs	Alencar *et al.*, 2020 [37]
	SMCs, ECs	Slenders *et al.*, 2021 [51]
	SMCs, ECs	Alsaigh *et al.*, 2022 [52]

miR, microRNA; NCD, normal cholesterol diet; SMC, smooth muscle cell; WD, western diet.

†This study additionally performed a trajectory analysis

*This study additionally performed a communication analysis

Overall, scRNA-seq efforts are in agreement that murine and/or human plaques comprise one or several vascular SMC subclusters with contractile characteristics (*Myh11*/*MYH11*, *Acta2*/*ACTA2*).[33,35,37,40,46,47,50,58,59] Each of these studies additionally reports one or multiple distinct SMC or SMC-derived subpopulations characterized by a loss of SMC markers, although the subpopulations in question markedly differ between studies. A recent meta-analysis of multiple scRNA-seq datasets of SMC lineage-traced atherosclerotic mouse models[33,35,37,40] provides more insight into the (dis)similarity of these SMC(-derived) subpopulations.[58] From this analysis, Conklin *et al.* conclude that a convergent finding is the presence of an SMC-derived intermediate cell state termed 'SEM' (stem cell, endothelial cell, monocyte).[58] Pan *et al.* previously reported a loss of contractile features and activation of ECM-related pathways (*Fn1*, *Col1a2*) in SEM cells and proposed that they represent an intermediate SMC phenotypic switching state.[35] Furthermore, Conklin *et al.* found strong evidence of SMC-derived macrophage-like cells, although there was a wide quantitative range between studies, likely due to technical differences.[58] Consistent with this, a recent study identified four phenotypically modulated SMC clusters in mouse atherosclerotic aortas, including a cluster of macrophagic calcific SMCs (*Gdf10*, *Hsd11b1*, *Pex5l*).[46] The other SMC subpopulations exhibited a mesenchymal chondrogenic (*Ifi27l2a*, *Gata4*, *Meox1*), an inflammatory and ECM regulation (*Pf4*, *Chad*, *Slc10a6*), or an inflammatory (*Ccl7*, *Ptx3*, *Ccl11*) phenotype.In the meta-analysis of Conklin *et al.*[58], a comparison with scRNA-seq data of human carotid lesions[35] showed many similarities, favoring the continued use of mouse models in atherosclerosis SMC research. In line with this, Depuydt *et al.* report a synthetic SMC cluster in human plaques that was derived from the plaque cap as suggested by the downregulation of typical SMC markers and the upregulation of ECM genes (*COL1A1*, *MGP*, *COL3A1*).[50] A small subset of this cluster was *KLF4*$^{+}$, which is indicative of differentiation into a more synthetic or macrophage-like phenotype. Additionally, Alencar *et al.* found that *KLF4*/*Klf4* regulates SMC transition to several phenotypes in advanced human carotid and murine plaques, including an *Lgals3*$^{+}$ osteogenic phenotype (*Runx2*, *Sox9*, *Cytl1*) that is likely detrimental for late-stage atherosclerotic lesion pathogenesis.[37] Similarly, Wirka *et al.* showed that *TCF21*/*Tcf21* regulates SMC phenotypic modulation in atherosclerotic lesions of human coronary and mouse arteries, specifically the transformation of SMCs into fibroblast-like cells termed 'fibromyocytes' (*Fn1*, *Lum*).[33] Another scRNA-seq study identified SMCs with enhanced calcification and ECM remodeling (*SPP1*, *IBSP*) in the atherosclerotic core versus SMCs with pro-inflammatory signaling (*C3*, *PLA2G2A*) in the proximal adjacent region of human carotid plaques.[52] Re-analysis of this dataset by Zhang *et al.* revealed SMC-derived *CD68*^{high}*ACTA2*^{low} macrophage-like cells enriched in phagocytic, inflammation, and cholesterol metabolism functions.[59]

Macrophages

An established hallmark of atherosclerosis development and progression is the accumulation of macrophages within the arterial intima, most of which are derived from monocytes that infiltrate the intima and subsequently differentiate into macrophages. The importance of monocyte recruitment for plaque progression was confirmed by Williams *et al.*, who combined scRNA-seq with fate mapping to reveal the limited proliferation capacity of specialized aortic intima resident macrophages (Mac^{AIR}) in murine aortic lesions.[36] Although this Mac^{AIR} population (*Itgax*, *Cx3cr1*, *Csf1R*) gave rise to the earliest foam cells in plaques, they were replaced entirely by recruited monocytes upon prolonged hypercholesterolemia. Still, the debate has long prevailed as to what macrophage populations are present in atherosclerotic lesions. The four most consistent macrophage phenotypes across scRNA-seq studies in mice include a resident-like macrophage (i), an inflammatory macrophage (ii), a triggering receptor expressed on myeloid cells-2 (Trem2) macrophage (iii), and an interferon (IFN)-inducible macrophage (IFNIC) (iv). The population representing resident-like macrophages (*Lyve1*) was consistently found in both healthy[28,31,60] and atherosclerotic[28,31,32,34,45,60] mouse aortas. One of these studies additionally revealed this subtype to highly express *Ccl24*, thereby promoting the trans-differentiation of SMCs to osteogenic-like cells in atherosclerosis.[45] All remaining populations were specific to atherosclerosis, including inflammatory macrophages (*Il1b*, *Cxcl2*)[28,60], otherwise referred to as non-foamy[32] or chemokine^{high} [34] macrophages. Trem2 macrophages, characterized by an upregulation of *Trem2*[28,32,34,60], were first described by Cochain *et al.*[28] and later shown to be identical to the lipid-laden foamy macrophages identified by Kim *et al.*[32] in diseased mouse aortas.[61,62] Furthermore, Kim *et al.* showed that Trem2 foamy macrophages are not pro-inflammatory, thereby resolving controversy in the field.[32] Of note, microRNA-33 (miR-33) silencing has been shown to reduce intraplaque inflammatory and Trem2 macrophage levels.[44] Silencing of miR-33[44] or netrin-1[43] additionally promotes atherosclerosis regression in part by altering macrophage transcriptomic profiles, either by upregulating genes associated with lipid metabolism (*Abcba*, *Ncoa1/2*) and chromatin remodeling/transcriptional regulation (*Brwd3*, *Ddx5*, *Hipk1*)[44] or by upregulating pathways involved in phagocytosis and migration (*Ccr7*).[43] The fourth macrophage population comprises IFNICs[32,34,60], which are enriched in IFN-inducible genes (*Ifit3*, *Irf7*, *Isg15*). While this subset was initially not found by Cochain *et al.*[28] and Winkels *et al.*[31], most likely due to the relatively small number of sequenced macrophages in these studies, a meta-analysis by Zernecke *et al.*[60] later revealed small numbers of IFNICs in these datasets. Moreover, this analysis verified all four previously established macrophage subsets in mice and expanded over the original studies by identifying a new subset resembling cavity macrophages (*Cd226*, *Itgax*).

The same group recently integrated scRNA-seq data of mouse and human atherosclerosis in an attempt further to refine the nomenclature of mononuclear phagocytes, including macrophages, and compare their transcriptomic signatures between mice and humans.[63] Integration of mouse data identified subpopulations with distinct gene expression profiles within the resident-like (*Cd209*^{high} and *Cd209*^{low}), inflammatory (*Ccr2*^{int}*MHCII*$^{+}$ and *Nlrp3*^{high}*Il1b*^{high}), and foamy (Mac^{AIR}, *Trem2*^{high}*Slamf9*, and *Trem2*^{high}*Gpnmb*) macrophage populations. In addition, cross-species comparison and data integration indicated conserved transcriptomic features of macrophages in mouse and human atherosclerosis. In particular, gene signatures of four major mouse macrophage subsets, i.e., resident-like, inflammatory, foamy/Trem2, and IFNIC macrophages, mapped to specific populations in human lesions. However, a subset of cavity macrophages (*Itgax*, *Cd226*) was detected only in mice, emphasizing that some, but not all, macrophage subclasses identified in mouse models are relevant to human atherosclerosis and should therefore be investigated further. Depuydt *et al*. found a different discrepancy between mice and humans, namely no significant overlap between human *IL1B*$^{+}$ pro-inflammatory macrophages and any macrophage populations present in mouse atherosclerotic plaques.[50] They did establish the presence of resident-like, inflammatory, and foamy TREM2 macrophage populations in human lesions, and additionally revealed resident-like macrophages to be pro-inflammatory with an upregulation of *TNF*. Moreover, the TREM2 foam cell-like population expressed smooth muscle actin (*ACTA2*), suggesting a transition from a macrophage to an SMC phenotype or vice versa, and displayed a fibrosis-promoting phenotype. While Fernandez *et al*. identified five distinct macrophage clusters in human carotid artery plaques, they functionally characterized only four.[49] Three subsets displayed activated (*HLA-DRA*, *CD74*) and pro-inflammatory (*CYBA*, *S100A9/8*, and *JUNB*, *NFKBIA*) states. The second of these macrophage populations (*CYBA*, *S100A9/8*) was enriched in TIMP metallopeptidase inhibitor 1 (*TIMP1*) and may therefore promote plaque stabilization by limiting ECM degradation. The final population showed a foam cell-like gene expression profile (*APOC1*, *APOE*) with, unsurprisingly, reduced pro-inflammatory signaling (*IL1*, *IFN*).

T cells

Even though atherosclerosis was traditionally perceived as a macrophage-dominated pathology, it has an immune component primarily involving T cells that was established in part due to scRNA-seq experiments. The accumulation of T cells in atherosclerotic lesions and the vascular adventitia was first observed through immunohistochemistry, according to which T cells comprised approximately 7.7-22% of all cells in human plaques depending on the region.[64,65] However, using scRNA-seq Fernandez *et al*. revealed that T cells in fact account for the majority of human

plaque immune cells.[49] Similarly, Depuydt *et al.* found that T cells comprised the majority (52%) of all cells analyzed from human lesions.[50] scRNA-seq studies in mice showed a somewhat smaller fraction of T cells, namely approximately 25% of all leukocytes in atherosclerotic aortas.[28,31] Of note, the apparent discrepancy between immunohistochemistry and scRNA-seq findings may partially be attributed to the enzymatic tissue digestion that precedes scRNA-seq. This may cause the loss of fragile cell types, such as macrophages, resulting in an overestimation of more durable cellular subsets, including T cells. Nonetheless, through histological analysis, Depuydt *et al.* confirmed that $CD3^+$ T cells indeed outnumbered $CD68^+$ macrophages in human plaques.[50]

Furthermore, scRNA-seq has uncovered considerable T-cell heterogeneity in atherosclerosis (reviewed in [66]). Overall, there are four consistent T-cell populations in murine atherosclerosis, namely $CD8^+$ cytotoxic T cells (i), naive T cells (ii), thymocyte-like T cells (iii), and multilineage-committed $CD4^+$ T cells expressing *Cxcr6* (iv). An integrative analysis of scRNA-seq data additionally identified a distinct cluster of regulatory T cells (T_{regs}) (*Tnfrsf4/18*, *Cd134*, *NT5e*).[60] $CD8^+$ cytotoxic T cells (*Cd8a/b*, *Ccl5*, *Gzmk*) were consistently present in healthy and diseased aortas but not in the adventitia.[15,28,31,60] Conversely, the other T-cell populations were atherosclerosis-specific and were also detected in the aortic adventitia. This includes a population of naive $Cd28^+Ccr7^+$ T cells, which additionally expressed *Lef1*, *Dapl*, and *Tcf7*.[15,28,31,60] The third cluster comprised T cells enriched in both CD4 and CD8 and was previously termed mixed.[28,31] It is important to note that due to the technical limitations of scRNA-seq, the presence of $CD8^+CD4^+$ T cells was not confirmed on a single-cell level. I.e., it was only proven that the cluster expressed both of these markers, not that the individual cells that comprise this cluster did so. Zernecke *et al.*[60] propose that this population represents immature T cells, and Winkels and Wolf[66] note that these cells indeed expressed the thymocyte genes *Tcf7*, *Rag1*, and *Ccr9*.[28,31] The final cluster of multilineage-committed $CD4^+$ T cells (*Cxcr6*, *Rora*, *Tmem176*)[15,28,31] was termed $Il17^+Cxcr6^+$ in the meta-analysis by Zernecke *et al.*[60] This population displayed a mixed T_{reg}-helper T (T_H)1-T_H17-T_H2 transcriptomic profile that differs markedly between studies, as discussed in more detail in the review by Winkels and Wolf.[66] They additionally propose that $Cxcr6^+$ T cells may be specific for apolipoprotein B (ApoB) based on overlapping gene signatures with ApoB-reactive T cells detected in mouse atherosclerosis.[41] These ApoB-reactive T cells displayed mixed T_{reg} and T_H1-T_H2-T_H17-follicular helper T (T_{FH}) transcriptional programs and support the notion that atherosclerosis has an autoimmune component. Integrative analysis of these datasets is, however, not available. Both Wolf *et al.*[41] and another study that identified a mixed T_{reg}-T_H phenotype in atherosclerotic mice[67] showed that these cells failed to protect

from atherosclerosis in adoptive transfers despite their T_{reg} origin. In line with this, Sharma *et al.*[42] found distinct gene signatures of T_{regs} in progressing versus regressing plaques in mice. In particular, T_{regs} in regressing plaques lacked *Nrp1*, a marker for thymus-derived or natural T_{regs}, suggesting they are induced in the periphery. Furthermore, scRNA-seq recently revealed that miR-33 silencing increases T_{reg} levels in murine plaques while reducing the accumulation of $CD8^+$ T cells and T_H1 cells.[44]

In contrast to the traditional T_H subsets in mice, two scRNA-seq studies of human atherosclerotic lesions report T-cell phenotypes primarily defined by their activation status.[49,50] Depuydt *et al.* identified a diverse landscape of activation-based $CD8^+$ and $CD4^+$ T-cell subclasses that varied from cytotoxic (*GZMA*, *GZMK*) to more quiescent (*LEF1*, *SELL*).[50] Similarly, Fernandez *et al.* found distinct T-cell subsets with gene signatures of T-cell activation (*NFATC2*, *FYN*), cytotoxicity (*GZMA*, *GZMK*), and exhaustion (*EOMES*, *PDCD1*) in plaques.[49] Conversely, blood T cells of the same patients displayed transcriptomic profiles associated with a resting phenotype (*KLF2*, *TXNIP*). Additionally, this study reports distinct differences in plaque T cells between asymptomatic and symptomatic patients, including an unexpected pro-inflammatory signature (*IFNG*) in the former. It remains unclear how T-cell populations in mice and humans overlap, highlighting the need for integrated cross-species analysis of scRNA-seq data.

B cells

With regard to B cells, scRNA-seq efforts have established that this cell type is less prevalent and displays less phenotypic heterogeneity than T cells in atherosclerotic plaques. Yet, it is important to consider that B cells predominantly populate artery tertiary lymphoid organs in the adventitia adjacent to advanced plaques.[31,68] Hence, scRNA-seq studies excluding the adventitial compartment are likely to find a smaller abundance of B cells in atherosclerotic vessels. This almost certainly applies to all studies with carotid endarterectomy patients given that this procedure involves the removal of the intima with the plaque from the artery without affecting the tunica media or adventitia. A meta-analysis by Zernecke *et al.*[60] of several scRNA-seq studies in mice[28,31,32,34] defined two B-cell clusters, namely a small cluster of B1-like cells and a larger cluster of B2-like cells that was also detected in healthy aortas.[28,31] Transcriptional markers that differentiated the B1- and B2-like subsets included, respectively, genes associated with B1 cells (*Tppp3*, *S100a6*, *Cd9*) and with germinal center and marginal zone B cells (*Fcer2a*, *Cd23*). In human lesions, Depuydt *et al.*[50] and Fernandez *et al.*[49] both detected one small, homogeneous B cell cluster. Fernandez *et al.* additionally showed that B cells, as expected, have a higher frequency in blood than in plaques.[49]

Neutrophils

Despite their small numbers, neutrophils play an important role in atherosclerosis and thrombosis.[69] In an integrated analysis of scRNA-seq data in mice, Zernecke *et al.*[60] found only a few neutrophils in multiple datasets of healthy[28,31] as well as diseased[28,31,32,34] aortas. Still, several of the individual studies failed to identify a neutrophil cluster[31,32], and to our knowledge, no scRNA-seq study to date has been able to detect a neutrophil population in human plaques.[49,50] Zernecke *et al.*[60] propose two intrinsic obstacles as potential explanations, namely the relatively low mRNA content of neutrophils and the degradation of RNA by the potent, readily releasable ribonucleases in which neutrophils are rich. The process of tissue digestion likely also poses a considerable problem. Despite these hindrances, Vafadarnejad *et al.* recently observed two distinct *Siglecf*^{high} and *Siglecf*^{low} neutrophil populations in mouse atherosclerosis that resembled those present in the murine heart after myocardial infarction.[38]

Aortic aneurysm

Single-cell transcriptomics has substantially contributed to mapping the cellular landscape of AAs. **Table 2** provides an overview of scRNA-seq studies performed in AA research. As with atherosclerosis, most experiments were conducted using mouse models due to difficulties in obtaining and processing human AA tissue. Of note, the field of experimental aneurysm research employs diverse modes of promoting AA in mice, including Angiotensin (Ang) II infusion[70], periaortic exposure to calcium chloride ($CaCl_2$)[71], elastase perfusion[72], and an MFS (Fbn1$^{C1041G/+}$) mouse model[73].

Table 2. Single-cell transcriptomics studies in aortic aneurysm tissue.

Sex	Genotype	Tissue
Mouse		
Unknown	*ApoE*$^{-/-}$	Abdominal aorta
Male and female	C57BL/6J, *Sting*$^{gt/gt}$	Ascending aorta
Male and female	*Fbn1*$^{C1041G/+}$	Aortic root/ascending aorta
Unknown	*TGFβR2*$^{iSMC\text{-}Apoe}$	Ascending aorta
Male	C57BL/6J	Infrarenal abdominal aorta
Male	*ApoE*$^{-/-}$	Suprarenal abdominal aorta
Male	C57BL/6J	Infrarenal abdominal aorta
Male	*Mef2c-Cre +/0*; *ROSA26R*$^{mT/mG}$	Ascending aorta
Male	C57BL/10	Ascending aorta/aortic arch
Male	*ApoE*$^{-/-}$	Suprarenal abdominal aorta
Male and female	*Fbn1*$^{C1041G/+}$; *Nkx2-5*$^{IRES\text{-}Cre}$; *Rosa*$^{tdTomato/tdTomato}$	Aortic root/ascending aorta
Human		
Male	MFS	Aortic root
Male and female		ATAA
Male and female		AAA
Male and female	MFS	Aortic root/ascending aortic aneurysm

AAA, abdominal aortic aneurysm; Ang II, angiotensin II; ATAA, ascending thoracic aortic aneurysm; BAPN, β-aminopropionitrile; $CaCl_2$, calcium chloride; CHD, chow diet; EC, endothelial cell; HCHFD, high-cholesterol high-fat diet; HFD, high-fat diet;

Endothelial cells

Even though endothelial dysfunction is an established contributor to AA formation and development, only a few scRNA-seq studies have comprehensively characterized EC populations in AA tissues. Li *et al*. identified two EC subclusters in healthy adult mice and an Ang II-induced mouse model of AAA, namely 'normal' ECs (*Pecam1*) and lymphatic ECs (*Pecam1*, *Lyve1*).[76] EC dysfunction in AAA was characterized by an upregulation of genes associated with EC proliferation as well as leukocyte migration and activation, the latter suggesting a role of ECs in inflammatory cell recruitment during AAA formation. These findings are partially supported by a recent study that found three EC clusters in Ang II-induced AAA and sham mouse models.[79] Two of these clusters had similar expression patterns and upregulated genes related to proliferation and regeneration (*Eng*, *Kdr*, *Chd5*). Conversely, the third EC cluster had a distinct gene signature and was enriched in inflammation and damage-

Exposure	Cell type focus	References
WD 2 weeks + Ang II 4 weeks	Macrophages	Hadi *et al.*, 2018 [70]
HFD 5 weeks + Ang II 1 week (during last week)	SMCs, macrophages	Luo *et al.*, 2020 [74]
4/24 weeks	SMCs	Pedroza *et al.*, 2020 [73†]
HCHFD 0/1/2/4 months	SMCs	Chen *et al.*, 2020 [75]
Elastase-induced AAA 7/14/28 days	SMCs, monocytes/macrophages	Zhao *et al.*, 2021 [72]
Ang II 4 weeks	Macrophages, fibrocytes	Li *et al.*, 2021 [76†]
$CaCl_2$-induced AAA	SMCs, fibroblasts, macrophages	Yang *et al.*, 2021 [71]
Ang II 3 days	Fibroblasts, SMCs	Sawada *et al.*, 2022 [77†,*]
BAPN 7/14/21 days	Macrophages	Liu *et al.*, 2022 [78*]
Ang II 4 weeks	Fibroblasts, SMCs, immune cells, ECs	Weng *et al.*, 2022 [79†,*]
16 weeks	SMCs	Pedroza *et al.*, 2022 [80]
	SMCs	Pedroza *et al.*, 2020 [73]
	Immune cells, non-immune cells	Li *et al.*, 2020 [81†,*]
	Monocytes/macrophages	Davis *et al.*, 2021 [82†]
	SMCs, fibroblasts, ECs	Dawson *et al.*, 2021 [83*]

MFS, Marfan syndrome; SMC, smooth muscle cell; WD, western diet.
†This study additionally performed a trajectory analysis
*This study additionally performed a communication analysis

related genes (*Vcam1*, *Vwf*, *Icam1*). During the process of AAA, the cell numbers of this pro-inflammatory phenotype increased, whereas those of the proliferative EC phenotypes decreased. The latter is not in line with the increased expression of EC proliferative genes observed by Li *et al.*[76] during AAA development. While Dawson *et al.*[83] also identified three EC clusters in ascending AA tissues from MFS patients and in non-aneurysmal control tissues, they appear to be somewhat dissimilar to those reported in mice. The largest EC cluster represented a baseline EC population and was enriched in *POSTN*, quiescence markers (*IL33*, *CDKN1A*), and genes associated with innate immune responses. This cluster also upregulated stress genes (*ATF4*, *JUN*, *FOS*), although the authors attributed this to artificial stress in response to tissue processing. The second-largest EC cluster represented a more activated EC phenotype termed 'healing ECs' and showed an upregulation of genes involved in angiogenesis (*FLT1*, *DLL4*, *NOTCH4*), collagen deposition, and remodeling. The smallest

cluster represented de-differentiated ECs and expressed genes associated with the coagulation pathway and gap junctions, as well as *COL1A2*, *CCND1*, and contractile genes. All EC clusters, in particular healing ECs, had an increased proportion in MFS compared to control tissues. By contrast, another study detected two EC subsets that were both decreased in ascending thoracic AA (ATAA) patients versus healthy controls.[81] Furthermore, one cluster (*SLC9A3R2*, *HLA-C*) exhibited higher cell-cell and cell-ECM junction scores than the other EC cluster (*CEMIP2*, *EMP1*), which is suggestive of lower cell motility and/or migration.

Smooth muscle cells

SMCs are widely considered the culprit cell type in the pathogenesis of AAs and have therefore been intensively studied in the context of this disease. scRNA-seq studies consistently report a loss of SMCs during AA formation in mice[71,72,75,76,78] and humans[81,84,85]. To our knowledge, the only exception is a recent study by Weng *et al.* that reports an SMC expansion in an Ang II-induced AAA mouse model.[79] Furthermore, several scRNA-seq experiments detected a distinct, frequently disease-enriched cluster of modulated SMCs.[73,78,80,81,83] Trajectory inference-based insights into SMC phenotypic switching in AAs will be further discussed below. In a study by Pedroza *et al.*, the principal distinction between MFS and control mouse aortic root/ascending aorta tissue was the presence of a disease-enriched subset of transcriptomically modulated SMCs with ECM organization- (*Fn1*, *Mgp*, *Eln*), proliferation-, and adhesion-related functions.[73] While modulated SMCs were not present in young Fbn1$^{C1041G/+}$ mice, the single SMC cluster observed in these mice upregulated genes associated with early SMC modulation, suggesting activation of this process. Despite finding similar patterns of SMC phenotypic switching in murine atherosclerosis, this study identified a transcriptomic profile specific to MFS (*Serpine1*, *Klf4*). scRNA-seq of an aortic root aneurysm sample from an MFS patient revealed modulated SMCs (*COL1A1*, *SERPINE1*, *TGFB1*) analogous to those found in adult MFS mice. The same group later identified a similar disease-enriched modulated SMC subtype (*Igfbp2*) in the same mouse model, together with quiescent SMCs (*Myh11*), *Tnnt2*$^{+}$ SMCs, and rare *Rgs5*$^{+}$ pericytes.[80] Consistent with their prior study[73], the SMC modulation signature was distinguished by highly expressed *Mmp2*, *Tgfb1*, and *Col1a1* and downregulated SMC contractile genes (*Cnn1*, *Myh11*, *Acta2*).[80] Pedroza *et al.* additionally investigated whether the embryologic origin of SMCs, either the second heart field (SHF) or the neural crest, influences their phenotypic switching.[80] Interestingly, although SMCs derived from both lineages underwent phenotypic modulation in the setting of MFS, they displayed different transcriptomic responses. In a β-aminopropionitrile (BAPN)-induced thoracic aortic aneurysm and dissection (TAAD) mouse model and control mice, Liu *et al.* found

13 SMC clusters that could be divided into *Rgs5^{low}* and *Rgs5^{high}* SMCs.[78] The latter represented phenotypically modulated SMCs and displayed an ECM remodeling (*Fn1*, *Mmp2*, *Col1a1*) and migratory transcriptomic profile similar to fibroblasts, with downregulation of SMC contractile markers (*Acta2*, *Mylk4*, *Myh11*). Notably, one *Rgs5^{high}* cluster was significantly expanded at the early stage of TAAD, while the other two were either expanded or contracted at the advanced stage.

Compared with Pedroza *et al.*[73], Li *et al.*[81] performed a more comprehensive scRNA-seq study on tissues from multiple ATAA patients and control subjects. They uncovered a cluster of fibromyocytes or modulated SMCs (*ACTA2*, *MYL9*, *COL1A2*) with a gene signature suggestive of high ECM production but not proliferation. Other SMC clusters included contractile SMCs (*ACTC1*, *ACTA2*, *MYL9*), stressed SMCs (*FOS*, *ATF3*, *JUN*), and two subsets of proliferating SMCs (*MGP*, *TPM4*, *MYH10*). Of note, the authors argue that the gene expression profile of the stressed SMC cluster may be induced by tissue dissociation and hence not truly represent its physiological molecular status. A study by Dawson *et al.*[83] conducted on multiple human MFS and control samples detected a similar cluster of fibromyocytes that expressed both contractile and ECM (*ELN*, *FLN1*, *VCAN*) genes and resembled the modulated SMCs described by Wirka *et al.*[33] in atherosclerosis. They additionally identified a de-differentiated, proliferative SMC phenotype (*CCND1*, *THY1*) increased in MFS tissues and speculated that this cluster may be similar to the modulated SMC cluster unique to MFS identified by Pedroza *et al.*[73]. Other SMC subtypes included mature contractile (*SMTN*, *CNN1*) and contractile SMCs (*MYH11*), stressed SMCs (*JUN*, *FOS*, *ATF3*) likely reflecting the technical artifacts of tissue processing, and intermediate SMCs with a similar gene signature as modulated SMCs but a higher expression of contractile genes. Furthermore, based on a consistent downregulation of the canonical transforming growth factor beta (TGFβ) pathway (*TGFBR2*) in SMCs, Dawson *et al.*[83] proposed that impaired TGFβ signaling contributes to disease pathology. This is consistent with the downregulation of major TGFβ receptors (*Lrp1*/*LRP1*, *Tgfbr2*/*TGFBR2*) in SMCs of Ang II-infused mice as well as human TAAs.[77]

Other studies found alternative evidence of SMC phenotypic transformation. For instance, Weng *et al.* observed a functional transformation of SMCs from a contractile to a secretory phenotype in an Ang II-induced AAA mouse model.[79] Both SMC clusters identified in this study expanded during AAA formation and upregulated secretory genes (*Spp1*, *Mgp*, *Eln*), while the expression of contractile genes (*Acta2*, *Cald1*, *Myh11*) was largely unaffected. Similarly, Li *et al.* report SMCs enriched in ECM-associated functions suggestive of a phenotypic transformation in response to Ang II infusion

in mice.[76] Another study found transcriptomic evidence of an SMC phenotypic switch from contractile to synthetic in the early phase of murine AAA.[71] The contractile SMC population (*Myh11*, *Acta2*, *Tagln*) was diminished in AAA, whereas the synthetic SMC cluster (*Acta2*, *Aqp1*), which highly expressed cell cycle-related genes, was increased. In contrast, Sawada *et al*. found that SMCs in Ang II-infused mice upregulated contractile genes (*Acta2*, *Cnn1*, *Tagln*) and ECM components (*Eln*, *Col1a1/2*, *Col4a1*), and downregulated proliferation genes (*Klf4*, *Egr1*).[77]

AA scRNA-seq experiments also revealed SMC gene signatures associated with stress response pathway activation and inflammation. Consistent with SMC depletion being a hallmark for AA, Li *et al*. found an upregulation of apoptotic signaling pathway regulation in SMCs of Ang II-induced AAA mice.[76] In another study, transcriptomic profiling highlighted the contribution of excessive activation of stress-responsive (*Egr1*) and Toll-like receptor (TLR) signaling (*Atf3*) pathways in SMCs at the onset of TAAD in mice.[78] Luo *et al*. induced sporadic AAD in mice with a combination of a high-fat diet and Ang II.[74] Aortic challenge induced *Sting* expression in two SMC clusters, accompanied by an upregulation of key genes associated with inflammation (*Il1b*, *Il6*, *Tnf*) and cell death via, e.g., apoptosis (*Casp3/7/9*, *Bak1*, *Bax*). Knockdown of *Sting* prevented this transcriptomic reprogramming, suggesting a crucial role for *Sting* in activating stress response and cell death pathways implicated in AA formation. Immunostaining of human sporadic ascending TAAD tissues suggested a similar activation of the stimulator of interferon genes (STING) pathway in SMCs. In healthy and elastase-induced aneurysmal infrarenal abdominal aortas (IAAs) of mice, Zhao *et al*. detected a small inflammatory-like SMC cluster (*Sparcl1*, *Thbs1*, *Notch3*) that proportionally increased during AAA progression, along with an upregulation of pro-inflammatory genes (*Cd68*, *Cxcl1/2*, *Il1r1*).[72] The other SMC subpopulations, namely quiescent-contractile SMCs (*Myh11*, *Acta2*, *Tagln*), proliferative-contractile SMCs (*Fos*, *Jun*, *Klf2/4*), and de-differentiated SMCs (*Mt1/2*, *Hk2*, *Gata6*), all decreased in AAA, accompanied with downregulation of contractile genes. Lastly, re-analysis of the human ATAA scRNA-seq dataset of Li *et al*.[81] revealed 16 distinct SMC clusters, which were further classified according to their canonical definitions into contractile, secreting (*COL1A1/2*), mesenchymal-like (*CD34*, *PDGFRA*), adipocyte-like (*FABP4*, *EBF2*), macrophage-like (*CD14*, *CD68*), and T-cell-like (*CD3D/G*) SMCs.[84] Only the contractile SMC phenotype was reduced in ATAA, whereas all other SMC phenotypes were significantly increased. All in all, scRNA-seq efforts in AA research have provided considerable evidence of SMC phenotypic switching commonly characterized by enhanced ECM regulation, as well as of increased stress-responsive and inflammatory signaling by SMCs.

Fibroblasts

Although a proportional decrease of fibroblasts is typically observed in AA using scRNA-seq[71,72,76,79,81,84,85], fibroblast heterogeneity plays a pivotal role in AA.[79] Nevertheless, there is no consensus on the number of fibroblast clusters in healthy aortic and AA tissue. As with SMCs, Pedroza *et al.* identified a different number of fibroblast clusters in young versus adult Fbn1$^{C1041G/+}$ mice, the difference being a distinct *Ly6a*$^{+}$ fibroblast cluster only detected in adult mice but not in young mice or an MFS patient.[73] Re-analysis of the adult mice scRNA-seq dataset by Zhou *et al.* revealed an additional, disease-specific *Spp1*$^{+}$ subpopulation of fibroblasts associated with enhanced ECM regulation.[86] However, other scRNA-seq studies report a decrease in ECM regulation in AAs along with an increase in proliferation. For instance, Ang II infusion induced a unique SHF-derived fibroblast subcluster in the ascending aorta of mice, characterized by downregulation of genes related to ECM components (*Col4a3*, *Eln*) and TGFβ signaling (*Tgfb2*, *Tgfbr2*) and upregulation of proliferative genes (*Cdk1*, *Mki67*, *Cks2*).[77] Another study observed a proportional decrease of quiescent fibroblasts that played a role in ECM turnover and collagen synthesis (*Dcn*, *Lum*, *Col1a1*) in an early-stage AAA mouse model compared with sham.[71] The second fibroblast cluster identified in this study had an inflammatory and proliferative gene signature (*Pclaf*, *Mki67*) and was slightly increased in AAA tissues. Aside from ECM modulation and proliferation, scRNA-seq studies have identified AA-associated fibroblast subtypes with various other functions. For example, Weng *et al.* identified 10 distinct fibroblast clusters in Ang II-induced AAA and sham mouse models, four of which increased in amount during the formation of AAA.[79] The expanded populations were defined as antigen-presenting (*Cd74*, *H2-Ab11*), activation (*Acta2*, *Pdgfrb*), tissue-repair (*Fn1*), and vascular (*Des*) fibroblasts. Enrichment analysis suggested that fibroblasts mainly participate in the AAA process through ECM metabolic homeostasis, but also via pathways such as oxidative phosphorylation and PI3K-Akt signaling. Similarly, Li *et al.* found a population of myofibroblasts (*Cd34*$^{-}$*Acta2*$^{+}$) involved in wound healing that increased significantly in AAA compared to healthy mouse tissues.[76] Other fibroblast clusters detected in this study included inactivated fibroblasts (*Cd34*$^{+}$*Acta2*$^{-}$) and an intermediate population, which were specifically enriched in the functions 'transmembrane receptor protein serine/threonine kinase signaling pathway' and 'connective tissue development', respectively.

Only two scRNA-seq studies provide an in-depth characterization of fibroblast populations in aneurysmal human aortic tissue.[81,83] Some, but not all, of their findings are comparable to those in mouse models, e.g., quiescent, proliferative, and healing gene signatures. Li *et al.* found two clusters of fibroblasts in both control and aneurysmal human ascending aortas.[81] One cluster upregulated *FBN1*

and proteoglycans (*LUM*, *DCN*), while the other highly expressed *ELN* and displayed higher cell-cell and cell-ECM junction scores. The former resembles the population of quiescent fibroblasts detected by Dawson *et al.* in both ascending AA tissues from MFS patients and control tissues.[83] This population showed an increased expression of genes related to fibroblast quiescence and ECM maintenance (*C1R/S*, *LUM*, *DCN*) and was additionally enriched in *ADAMTS* genes, which were previously linked to AA formation. As with ECs and SMCs, Dawson *et al.* also identified stressed fibroblasts (*CXCL2/3*, *IER3*, *ATF3*) that likely represented fibroblasts in a stressed state due to tissue processing rather than a truly distinct phenotype.[83] The final cluster was consistent with activated fibroblasts and displayed a proliferative and healing gene signature, with an increased expression of genes involved in, e.g., SMC contraction (*MYL9*, *TAGLN*, *ACTA2*) and angiogenesis (*NOTCH3*, *THBS2*, *COL18A1*). Only this population showed an increased fraction in MFS patients.

Macrophages

A principal feature of the pathogenesis of AA is the accumulation of immune cells, particularly macrophages, as confirmed by single-cell transcriptomics.[71,72,75,76,78,79,81,84,85,87] One common scRNA-seq finding with regard to macrophages is an AA-associated upregulation in pro-inflammatory signaling. Zhao *et al.*, for instance, found a cluster of inflammatory macrophages (*Ccl2*, *Il1b*, *Mmp9*) in IAAs of elastase-induced AAA mice and healthy controls.[72] Other subpopulations included two types of aortic-resident macrophages (*Cx3cr1*, *Flt3*), blood-derived monocytes (*Adgre1*, *Itgam*, *H2-Aa*), and blood-derived reparative macrophages (*Arg1*, *Egr2*, *Il1r2*). Elastase exposure induced significant expansion of all clusters except for *Cx3cr1*$^{+}$ resident macrophages. In a $CaCl_2$-induced mouse model of early-stage AAA and in control mice, Yang *et al.* identified a macrophage population with a similar inflammatory transcriptomic profile (*Il1b*, *H2-Ab1*).[71] Unlike in Zhao *et al.*[72], resident-like macrophages did not form a distinct cluster but were concentrated in an anti-inflammatory (*Pf4*, *Mrc1*) and a proliferating (*Stmn1*, *Mki67*) macrophage cluster.[71] Inflammatory macrophages were responsible for the observed accumulation of macrophages in an early stage of AAA development, while the importance of local macrophage proliferation in AA has yet to be proven. Through an integrated analysis of scRNA-seq data of both aforementioned studies[71,72], Cheng *et al.* uncovered four gene modules positively associated with AAA primarily functionally enriched in collagen-containing ECM, myeloid leukocyte migration, and the PI3K-Akt signaling pathway.[87] Additionally, *Clec4e*, *Il1b*, and *Thbs1* were identified as key monocyte/macrophage-related genes, and a high expression of *CLEC4E* and *IL1B* was discovered in the human AAA dataset of Davis *et al.*[82] Interestingly, in their original study, Davis *et al.* discovered that the activation of an inflammatory immune response (*REL*, *TNF*, *NFKB1*) can at least partly be attributed to a significant

enrichment of the histone demethylase *JMJD3* in macrophages and monocytes.[82] By integrating human TAA scRNA-seq data[81,88], Wang *et al.* revealed macrophages predominantly enriched in immune-related pathways such as antigen processing and presentation and T_H-cell differentiation.[85] Some of these pathways were also identified by bulk scRNA-seq, suggesting that macrophages are largely responsible for inducing inflammatory responses involved in TAA formation and progression.

Of note, although some studies determine the expression of traditional M1 and M2 macrophage markers[72,76,79,81], this panel is not sufficiently specific to fully capture the functional heterogeneity observed in macrophages in AA. This is illustrated by a recent study in Ang II-induced AAA and healthy mice, which found six monocyte/macrophage clusters, including three clusters of M2-type macrophages (*Arg1*, *Cd163*, *Stab1*) that decreased during AAA progression.[79] Reportedly, macrophages were simultaneously polarized in an inflammation-promoting direction, although there was no apparent increase in M1 marker genes (*Cd68*, *Tlr2*, *Ciita*), which were expressed irregularly in the clusters. Li *et al.* did detect three specific M1-like clusters (*TNF*, *IL1B*, *NFKB1*) with inflammatory (*CCL3L1*, *CCL4*, *TNF*), tissue remodeling (*EREG*, *TIMP1*, *VCAN*), and $CD8^+$ T cell-presenting (*HLA-F*) functions in human ATAA and control tissues.[81] They additionally found two M2-like clusters (*MRC1*, *STAB1*, *CD163*) with roles in glucose metabolism (*PDK4*), anti-inflammation, and phagocytosis, and clusters associated with the IFN response (*IFI44l*, *ISG15*, *IFIT1*), tissue remodeling (*IGFBP7*, *ADAMTS1*, *MMP2*), and proliferation (*H2AFZ*, *TUBB*, *CKS1B*). M1-like populations were proportionally increased in tissues of ATAA patients, whereas remodeling macrophages were decreased.

This decrease in tissue remodeling macrophages is not in line with several scRNA-seq studies that report AA-associated macrophages involved in maladaptive ECM degradation and tissue remodeling, e.g., through upregulation of matrix metalloproteinase (MMP) family proteins. Liu *et al.* identified three subpopulations of macrophages in TAAD and sham mouse models, i.e., $Lyve1^+$ resident-like, $Cd74^{high}$ antigen-presenting, and $Il1rn^+/Trem1^+$ pro-inflammatory macrophages, that were all significantly expanded at the advanced stage of TAAD.[78] Additionally, this study pinpoints the latter as the predominant source of most detrimental molecules that contribute to TAAD development, including MMPs and inflammatory cytokines. This finding was verified in human TAAD patients through bulk RNA-seq and is supported by another scRNA-seq study using a sporadic AAD mouse model.[74] Here, a $Sting^{high}$ macrophage cluster upregulated genes associated with both inflammation (*Il1b*, *Ccr2*, *Cxcr3*) and ECM degradation (*Mmp2/8/9*). This upregulation was prevented in $Sting^{gt/gt}$ mice. Conversely, another study found non-inflammatory

macrophages responsible for ECM degradation and remodeling.[76] In healthy mice and an Ang II-induced mouse model of AAA, Li *et al*. discovered three macrophage clusters, namely *Trem2*$^{+}$*Acp5*$^{+}$ osteoclast-like macrophages, *Mrc1*$^{+}$*Cd163*$^{+}$ M2-like macrophages, and *Il1b*$^{+}$*Ccr2*$^{+}$ M1-like macrophages.[76] Osteoclast-like macrophages were increased in AAA tissues, and both osteoclast-like and M2-like macrophages highly expressed MMPs and showed an upregulated expression of cathepsins in response to Ang II infusion.

T cells

Similar to macrophages, many scRNA-seq studies report a proportional increase in T cells in murine[72,76,78,87] and human[81,84,85] AA tissues. Conversely, Yang *et al*. found an unchanged percentage of T cells and other adaptive immune cells, including B cells, in early-stage murine AAA.[71] A possible explanation for this discrepancy is that the adaptive immune response has not yet been fully activated at this stage. However, another study identified fewer T and B cells in late-stage murine AAA compared to healthy mouse tissues.[79] Still, critical transcriptomic alterations occurred in T cells during AAA development, including significant upregulation of *Cd3* and *Cd8* while *Cd4* expression remained essentially unchanged. In addition, AAA-associated T cells were enriched in ribosomal protein synthesis and T-cell differentiation and activation mechanisms. Zhao *et al*. identified three T-cell subpopulations in elastase-induced AAA and control mice, one of which accounted for the majority of T cells (*Ly6c2*, *Cd8a*, *Cd8b1*) (T 1).[72] The other subpopulations were defined by *Gzma* (T 2), which is specific to cytotoxic T cells and NK cells, and *Cd4* and *Il2ra* (T 3), the latter of which is characteristic of T_{regs}. During the progression of AAA, the cytotoxic T and NK cell-like cluster was proportionally increased on day 7 and restored on day 14. In contrast, the other two clusters were decreased on day 7 and restored on day 14. A different study in mice showed T cells to be enriched in the function of NK-cell activation (*Klrb1c*, *Il2rb*) in response to Ang II infusion.[76] In human ATAA tissues, Li *et al*. found T cells to be the largest cell population and detected a total of 11 distinct clusters that were all expanded compared to control.[81] Three of these clusters comprised CD4^{+} T cells, namely active CD4^{+} T cells (*CREM*, *CXCR6*, *GZMB*), resting CD4^{+} T cells (*CCR7*, *IL7R*, *CCL20*), and T_{regs} (*IL2RA*, *CTLA4*, *TNFRSF18*). In addition, two clusters of CD8^{+} T cells were identified, i.e., active CD8^{+} T cells (*GZMK*, *CRTAM*, *CCL4*) and CD8^{+} terminally differentiated effector T cells (*GNLY*, *PRF1*, *CCL5*). Other clusters consisting of both CD4^{+} and CD8^{+} T cells included heat shock protein (HSP) T cells (*JUN*, *FOS*, *HSPA1A/B*), GTPase of the immune-associated nucleotide-binding protein (GIMAP) T cells (*GIMAP1/4/7*, *MALAT1*), stressed T cells (*JUN*, *FOS*, *DNAJA1*), proliferating T cells (*TUBA1B*, *HMGB1*, *CKS1B*), switched T cells (*TAGLN*, *MYH11*, *TPM1*), and IFN

response T cells (*ISG15*, *IFI44*, *LY6E*). The authors argue that the HSP and IFN response subtypes may have resulted from tissue processing and viral infection, respectively. Finally, Wang *et al.* demonstrated that T cells in human TAA are enriched in pathways such as 'ribosome', 'antigen processing and presentation', and, curiously, 'lipid and atherosclerosis'.[85]

B cells

An AA-associated expansion of B cells is also commonly found by scRNA-seq studies in mice[76,78] and patients[81,84,85]. By contrast, Zhao *et al.* observed a contraction of the B-cell population in IAAs of elastase-induced AAA versus healthy mice.[72] Interestingly, Li *et al.* showed that B cells in aortic tissues from an Ang II-induced AAA mouse model upregulated genes associated with an immunoglobulin-mediated immune response (*Cr2*, *Ighm*), which the authors relate to activation to plasma cells.[76] This is supported by another study using the same mouse model that reports an increase in plasma cells during AAA formation in spite of a decrease in B cells.[79] In the AAA process, B cells were predominantly enriched in B-cell receptor signaling, ribosomal protein synthesis, and NF-κB signaling pathways. In agreement with both aforementioned studies[76,79], Li *et al.* found a considerably expanded population of plasma cells (*MZB1*, *IGHA1*, *SSR4*) in human ATAA compared to control tissues.[81]

Lineage tracing

Single-cell transcriptomics has not only contributed to elucidating the cellular composition of tissues, but also to reconstructing lineage relationships between cells (reviewed in [8,89,90]). Traditional lineage tracing involves the genetic labeling of a cell and subsequent tracking of its progeny in, for instance, mouse model systems. This technique has been widely applied in vascular pathology research, especially in the fields of atherosclerosis[33,35–37,39–41,46,91–93] and AA[75,80]. For instance, Pan *et al.* used genetic lineage tracing to track SMC trans-differentiation in atherosclerotic mice and demonstrated that SMCs differentiate into multiple cell types or states, including macrophage-like cells (*Lgals3*, *Cd52*) and fibrochondrocytes (*Fn1*, *Col1a1/2*).[35] Furthermore, Chen *et al.* generated SMC lineage-tracing mice to establish that abrogation of TGFβ signaling in SMCs combined with hypercholesterolemia induces AAs by reprogramming SMCs into macrophage-like cells as well as into mesenchymal-like stem cells (MSCs) (*Ly6a*, *Cd34*, *Cd44*), from which chondrocytes (*Sox9*), osteoblasts (*Runx2*), and adipocytes (*Ppar γ*) arise.[75]

Alternatively, lineage or differentiation trajectories can be inferred based on scRNA-seq data using various bioinformatics tools (reviewed in [94]). Such tools detect the pattern of a dynamic process, e.g., cell differentiation, and subsequently arrange the transcriptomes of individual cells according to their differentiation status. The majority of computational methods rely on dimensionality reduction for ordering cells along a dimension otherwise known as 'pseudotime'.[8] One of the most commonly applied algorithms that performs such pseudotemporal ordering is Monocle.[95,96] RNA velocity is an alternative technique that infers cell state trajectories based on the splicing dynamics of transcripts.[97] While transcriptome-derived lineage predictions provide more detailed phenotypic information than genetic lineage tracing, they do not necessarily reflect true genetic relationships between cells. Concepts and limitations of cell trajectory inference from scRNA-seq data are reviewed in Tritschler *et al.*[98] In the remainder of this section, we will provide an in-depth discussion of the knowledge of healthy and diseased vasculature owing to scRNA-seq-based trajectory analysis.

Healthy vasculature

Some of the rapidly increasing number of scRNA-seq efforts have generated insights into the lineage of arterial cells in healthy vasculature.[15,17,19,21,23,24] For example, Lukowski *et al.* inferred pseudotime trajectories from mouse scRNA-seq data to define an endothelial hierarchy wherein endovascular progenitor cells (*Pdgfra*, *Il33*, *Sox9*) transition to mature, differentiated endothelial cells (*Sox18*, *Pecam1*, *Fabp4*) in aortic endothelium.[21] In human cardiac (aortic, pulmonary, and coronary) arteries, trajectory analysis arranged vascular SMCs of four clusters into one principal trajectory.[17] The proliferative (*Mt1a/m*, *Fabp4*) and aorta- and pulmonary artery-specific synthetic (*Cytl1*, *Lum*) SMC clusters were located on opposite ends of the differentiation trajectory, with contractile (*Cnn1*, *Rgs5*) and coronary artery-specific synthetic (*Fn1*, *Vcan*) SMCs located roughly in between. Pseudotime analysis has also been employed for obtaining independent evidence for the putative topography of vascular cell subpopulations. For instance, in developing a single-cell transcriptome atlas of murine ECs, Kalucka *et al.* captured the anatomical topography of various EC subtypes alongside the vascular tree.[23] Aside from revealing a differentiation trajectory from arteries to veins via capillaries, this analysis established several markers of, e.g., large arteries (*Fbln5*, *Cytl1*), capillaries (*Rgcc*), and large veins (*Lcn2*) in the brain. In healthy mouse aortas, Yu *et al.* observed that two spatially distributed SMC clusters, namely *Vim*$^{+}$ and *Malat1*$^{+}$ SMCs located in the abdominal and thoracic segments of the aorta, respectively, also developed into opposite differentiation directions.[19]

Atherosclerosis

Vascular cells in atherosclerotic plaques have a tremendous potential for undergoing phenotypic switching, otherwise referred to as phenotypic modulation or de-differentiation. Cell types with considerable differentiation potential include ECs and SMCs. EC plasticity plays a crucial role in atherosclerosis progression by giving rise to plaque-associated mesenchymal cells through EndMT.[54] EndMT can be induced by disturbed blood flow, a major contributor to atherosclerotic plaque formation as illustrated by the preferential development of lesions in vascular segments that are curved or branched. Andueza *et al.* investigated the effect of pro-atherogenic disturbed flow on the transcriptomic profile of ECs through scRNA-seq and single-cell assay for transposase accessible chromatin sequencing (scATAC-seq) of mouse arteries following partial carotid ligation.[99] Trajectory inference analysis revealed extensive endothelial reprogramming, with transitions to not only mesenchymal cells (EndMT) (*Tagln*, *Cnn1*) but also to hematopoietic stem cells (endothelial-to-hematopoietic transition, EndHT) (*Sox7*, *Sox17*), endothelial stem/progenitor cells (*Cd157*, *Sca1*), and an unforeseen immune cell-like phenotype (EndICLT) (*C1qa/b*, *C5ar1*). In a similar scRNA-seq study using a partial carotid ligation mouse model, Li *et al.* found a mechanosensitive *Dkk2*^{high} EC subpopulation that possibly transformed from *Klk8*^{high} ECs under disturbed flow.[100] This *Dkk2*^{high} subpopulation was enriched in biological processes related to flow disturbance, including epithelial-to-mesenchymal transition (EMT) and TGFβ signaling. Another important driver of atherosclerosis that has been shown to trigger endothelial changes is arterial stiffness. Zamani *et al.* performed scRNA-seq of human coronary artery ECs cultured *in vitro* on both physiological and pathological stiffness substrates.[101] RNA velocity analysis showed an EndMT trajectory as well as an unexpected reverse mesenchymal-to-endothelial transition (Mes-to-EndT) that was blocked by high stiffness. This is consistent with a model in which the pro-atherogenic stiffness-induced accumulation of mesenchymal cells is, at least partly, attributable to a reduced conversion back to an endothelial state. Of note, lineage tracing has not only generated insights into EC de-differentiation in atherosclerosis but also into EC differentiation. By integrating lineage-tracing mouse models with pseudotime analysis of scRNA-seq data, Deng *et al.* uncovered that vascular *c-Kit*$^{+}$ stem/progenitor cells in healthy and diseased mouse aortas display endothelial differentiation potential.[93] *c-Kit*$^{+}$ cells contributed not only to endothelial turnover in atheroprone regions during homeostasis but also to endothelial replacement in transplant atherosclerosis. Different from 'regular' atherosclerosis, recipient immune-mediated reactions to donor ECs and SMCs constitute an underlying mechanism in transplant atherosclerosis.[102]

A number of scRNA-seq studies have characterized the trajectories and fates of SMC phenotypic switching given its established role in atherosclerosis.[55,56] In hypercholesterolemic mice, Chattopadhyay *et al.* found that contractile SMCs initially undergo uniform phenotypic modulation, followed by a separation into two distinct modulated SMC clusters (*Lcn2*, *Serpina3n*, and *Lars2*, *C3*).[47] The former subpopulation further diverged into two major lineages, while the latter formed a single lineage that was absent upon *Perk* deletion in SMCs. A recent meta-analysis of murine atherosclerosis scRNA-seq datasets by Conklin *et al.* highlights the power of combining genetic lineage tracing with single-cell transcriptomics.[58] This study inferred re-differentiation trajectories of SMCs through pseudotime analysis and removed uncertainty about cells truly being SMC-derived by performing this analysis on data from SMC lineage-positive cells. They uncovered a multitude of state transition paths, many of which traversed the SEM population (*Vcam1*, *Fn1*). This is in agreement with the notion proposed by Pan *et al.* that SEM cells represent a de-differentiated stem cell-like state that gives rise to other SMC-derived cell types.[35] Interestingly, one trajectory traversed from contractile SMCs (*Myh11*, *Acta2*) to macrophage-like cells (*Lgals3*, *Cd68*) through a proliferative cell subpopulation (*Plk1*, *Birc5*, *Ccna2*). Recent findings by Schlegel *et al.* partially support the presence of SMC-derived macrophages in murine atherosclerotic plaques.[43] Monocytes and *Cx3cr1*$^{-}$ macrophages remained distinct in a pseudotime trajectory, suggesting these macrophages might be derived from a non-myeloid cell origin. However, as *Cx3cr1*$^{-}$ macrophages were enriched in some vascular SMC markers (*Fn1*, *Tagln2*) but not in others (*Myh11*, *Acta2*), their origins remain unclear. In their meta-analysis, Conklin *et al.* also found links connecting SMCs, SEM cells, and fibroblast-like cells.[58] This is consistent with a recent study in which pseudotemporal ordering of a published scRNA-seq dataset of diseased human coronary arteries[33] revealed distinct derivations of fibroblast-like cells from SMCs.[10] Key alterations in gene expression along the transition path included upregulation of inflammation (*C3/7*, *CXCL12*) and ECM (*FBLN1*) genes and downregulation of genes associated with healthy SMCs (*MYH11*, *IGFBP2*, *PPP1R14A*). Lastly, in the study by Li *et al.* on flow effect, disturbed flow induced *Spp1*^{high} SMCs as indicated by their location at the end of the pseudotime trajectory.[100] Interestingly, these SMCs were enriched in genes associated with vascular stiffening (*Thbs1*), osteoblast differentiation (*Mef2c*, *Tnc*), and fibrosis (*Ctgf*, *Col5a1/2*), suggesting a role in arterial stiffness.

Aortic aneurysm

The concept of cellular phenotypic plasticity over a continuous spectrum is not specific to atherosclerosis but is characteristic of AA as well. Cell types that possess the potency to differentiate in AA include not only SMCs but also macrophages and fibroblasts. Through pseudotime trajectory analysis of transcriptomes, Pedroza *et*

al. inferred that modulated SMCs gradually trans-differentiate from SMCs in MFS mice.[73] Additionally, this analysis revealed differentially expressed genes with multiple patterns of gene modulation along the spectrum of phenotypic switching. For instance, some genes were activated relatively early in pseudotime (*Serpine1*, *Klf4*), whereas others peaked at the end of the trajectory (*Tnfrsf11b*, *Vcam1*). In human ATAA, Li *et al.* described two re-differentiation trajectories from contractile SMCs toward (i) inflammatory non-immune cells expressing macrophage-specific genes (*C1QA/B*) and remodeling macrophages (*IGFBP7*, *ADAMTS1*), and (ii) inflammatory non-immune cells showing a T cell-like gene signature (*CXCR4*, *CCL4*) and switched T cells (*TAGLN*, *MYH11*, *TPM1*).[81] Integrated trajectory analysis of this and another scRNA-seq dataset[88] provided more insights into cellular transitions of macrophages and T cells during the formation of human TAA.[85] Interestingly, macrophages and T cells experienced respectively ten and seven different subtypes or states from the normal aorta to TAA along with trajectory progression. Davis *et al.* performed pseudotime analysis of human AAA tissues and inferred a differentiation trajectory from one monocyte cluster to macrophages via another monocyte cluster.[82] The increase in *JMJD3* expression along this trajectory was accompanied by a higher expression of *TNFAIP3*, *IL1B*, and *NFKBIA*. Based on trajectory analysis, Li *et al.* proposed the re-polarization of *Trem2*$^{+}$*Acp5*$^{+}$ osteoclast-like macrophages and M2-like macrophages toward M1-like macrophages in AAA pathogenesis in Ang II-infused mice.[76] During the M2-to-M1 transition, several genes associated with this polarization either increased (*Pgam1*, *Pkm*) or decreased (*Cd36*, *Klf4*) in expression. Furthermore, although a disease-enriched population of *Cd34*$^{+}$*Col1a2*$^{+}$ bone marrow-derived fibrocytes was initially classified as a macrophage subtype, further pseudotime analysis revealed no strong connection between this cluster and other macrophage clusters. Instead, fibrocytes resembled inactivated fibroblasts and tended to differentiate toward fibroblasts and subsequently myofibroblasts. Similarly, another study reconstituted the pseudotime of fibroblasts to predict their transformation over the course of disease in an Ang II-induced AAA mouse model.[79] One fibroblast cluster was identified as the starting point and ultimately differentiated into antigen-presenting (*Cd74*, *H2-Ab11*) and tissue-repair (*Fn1*) fibroblasts. Crucial reprogramming genes were predominantly enriched in immune system regulation (*Cfh*, *Cd55*). Lastly, Sawada *et al.* employed trajectory inference to demonstrate that the previously mentioned Ang II-induced SHF-derived fibroblasts in mice were derived from a fibroblast cluster also present in healthy mice.[77]

Cellular crosstalk

Single-cell transcriptomics also offers the opportunity to decipher cellular communication (reviewed in 9). In particular, cell-cell interactions can be inferred from the coordinated expression of ligands and their cognate receptors. Numerous intercellular signaling pathways are documented in available databases of ligand-receptor pairs.[103] Based on scRNA-seq data, a communication score can be estimated to quantify the potential ligand-receptor interaction between one set of ligand-expressing cells and another set of receptor-expressing cells[9] A communication score is typically computed for each ligand-receptor pair by applying a scoring function, such as expression threshold or product, on the expression of the corresponding genes. In addition, various bioinformatic tools that employ advanced statistical methods for imputing cellular crosstalk from scRNA-seq data have rapidly emerged (reviewed in 104). CellPhoneDB[105] and CellChat[106] are the most widely used computational tools in vascular physiology and pathology research.[10,16,18,19,50,78,79,84–86,107,108] Unlike most methods for cell communication inference, CellPhoneDB and CellChat consider multisubunit protein complexes by assessing whether all subunits of multimeric proteins are simultaneously expressed.[9] This better represents functional ligand-receptor interactions, thereby decreasing the risk of false-positive predictions. Other approaches consider information beyond ligand-receptor pairs, as exemplified by NicheNet[109], a tool that employs a network method to incorporate downstream targets and thereby uncover the activation of intracellular signaling. However, physical proximity is key for most communicative links between cells, and information on the spatial position of cells is lost with scRNA-seq. PIC-seq represents a novel approach for describing physical interactions that combines cell sorting with scRNA-seq to acquire and transcriptionally profile physically interacting cells (PICs).[110] However, to the best of our knowledge, this technique has not yet been applied to study vascular systems. In this section, we provide an overview of the discoveries of healthy and diseased vasculature enabled by cell-cell communication profiling based on scRNA-seq.

Healthy vasculature

A number of scRNA-seq experiments have contributed to elucidating the cell-cell interactions governing vascular homeostasis.[15–19] In a recent study of healthy mouse aortas, one EC subset (*Vcam1*, *Ace*) and one SMC subset (*Malat1*) were located in the center of the cell-to-cell interaction network, suggesting they play a vital role in aorta physiology.[19] Using the CCInx tool[111], Hu *et al.* revealed that cellular crosstalk between vascular SMCs and fibroblasts was predominant in human cardiac arteries and mainly relied on ECM molecules (*COL3A1*, *DCN*, *FN1*).[17] Cell-cell communication between ECs and macrophages through cell surface proteins (*CD74*, *B2M*) and cytokines (*CCL2*,

IL1B) also contributed to maintaining arterial structure and function. Moreover, interactions between ECs and immune cells (*ICAM1*/*VCAM1-ITGB2*) were speculated to increase in atherosclerosis. Another scRNA-seq study profiled cellular communication in both healthy and at-risk vasculature.[16] This study constructed intercellular networks of mouse aortas under normal conditions or with high glucose levels, dietary salt, or fat intake. This analysis revealed strong stromal cell-EC, stromal cell-SMC, and EC-SMC correlations. The last two were considerably diminished in the at-risk aorta, along with decreased interactions of these three cell types within their own subtypes.

Atherosclerosis

Cell-cell communication profiling of plaques has provided potential mechanisms of how cells collaboratively give rise to and advance atherosclerosis. For example, Gu *et al.* discovered enhanced cell intercommunication between a mesenchyme cluster (*Cd248*, *Procr*) and inflammatory cells (*Col1a1-Cd44*, *Ccl2-Ccr2*), especially inflammatory macrophages (*Ms4a6c*, *Gngt2*), in the adventitia of apolipoprotein E (ApoE)-deficient mice.[15] This implies a role for mesenchymal cells in initiating adventitial inflammation in early-stage atherosclerosis. In contrast, through ligand-receptor interaction analyses, Kan *et al.* showed extensive crosstalk between ECs, SMCs, and macrophages in the ascending aorta of mice fed a high-fat diet.[18] This was predominantly facilitated by chemokine signaling (*Cxcl12-Ackr3*, *Ccl6-Ccr2*), suggesting that cell-cell communication drives vascular inflammation in this setting. Ma *et al.*[10] inferred cell-to-cell communication from diseased human coronary artery scRNA-seq data[33] using both CellChat[106] and the network-based modeling method scTalk[112]. This analysis suggested that fibroblasts exert strong signals in laminin (*LAMB2-CD44*) and complement (*C3-C3AR1*) signaling pathways in the plaque microenvironment.[10] In particular, fibroblasts signal to SMCs, T cells, and pericytes through pro-inflammatory and repair molecules such as complement component 3 (C3), fibulin-1 (FBN1), and MMP2. Interestingly, a significant upregulation of these molecules was observed along the SMC-to-fibroblast trajectory as discussed previously. Another study applied CellPhoneDB[105] to the same dataset and found significant ligand-receptor relationships between T-cell-expressed *AREG*, which encodes the cytokine amphiregulin, and its receptors on both SMCs (*ICAM1*, *EGFR*) and macrophages (*ICAM1*).[108] These findings suggest that T cells interact with the aforementioned cell types by secreting amphiregulin, thereby promoting SMC proliferation and irreversible plaque formation. Instead of a bioinformatic tool, Fernandez *et al.*[49] employed an established scoring function that computes the average of the product of ligand and receptor expression of two cell types across all single-cell pairs.[113] This approach was used for predicting cellular interactions that promote the distinct functional and activation states of T cells and/or macrophages (e.g., pro-inflammatory) observed

in atherosclerotic lesions of asymptomatic and symptomatic patients.[49] Overall, T cell-T cell and T cell-macrophage intercellular communications carefully controlled the specialized functions of T cells. A key finding was the predicted interaction of the same ligand *(IL-15*) with distinct receptors on T cells in asymptomatic (*IL15RA*) versus symptomatic (*IL2RB*) plaques, either promoting cell proliferation, migration, and apoptosis inhibition or regulating T_H1 reprogramming and differentiation, respectively. Furthermore, the analysis revealed that while the functional states of macrophages are in part self-regulated, they also rely upon signals from T-cell ligands that bind to, e.g., TLRs. In contrast, Depuydt *et al*. report a limited number of potential ligand-receptor interactions between immune cells in human plaques.[50] This may be a consequence of the absence of T-cell receptor-related genes in the interaction database. Furthermore, in contrast to Fernandez *et al*.[49], this study also included non-immune cells in the analysis which potentially present more prominent cellular crosstalk pathways in the plaque. Indeed, cell intercommunication at the lesion site was most prevalent between myeloid cells, ECs, and SMCs. Interactions between myeloid cells and ECs were primarily associated with chemotaxis (*CCL2/CXCL8-ACKR151*) and extravasation of myeloid cells (*CD44-SELE*, *SELL-CD34*).

Aortic aneurysm

Lastly, intercellular communication inference has enhanced our understanding of the cell-cell interactions that underlie aortic dysfunction and AA development. Cell-cell communication profiling in a BAPN-induced TAAD mouse model uncovered a potential role of macrophages in stimulating tumor necrosis factor (TNF)-mediated SMC apoptosis (*Tnf-Tnfrsf1a*) and the activation of TGFβ signaling in SMCs (*Tgfb1-Tgfbr1*) in early-stage TAAD.[78] In addition, SMCs interact with fibroblasts, macrophages, and ECs via *Fgf1*-mediated paracrine signaling. Through expression thresholding, in which ligand-receptor pairs are considered 'active' if both genes are expressed above a certain threshold, Sawada *et al*. demonstrated enhanced cell-cell interactions among SMC and fibroblast clusters under Ang II infusion in mice.[77] The previously discussed unique subcluster of SHF-derived fibroblasts especially had more potential interactions with another fibroblast cluster from which they originate and with SMCs.

A number of scRNA-seq studies report a role for secreted phosphoprotein 1 (Spp1) in cellular signaling in AA. For instance, analysis of Spp1-mediated cell communication in data of $Fbn1^{C1041G/+}$ mice suggested that Spp1 regulates fibroblast and SMC crosstalk predominantly through the receptor *Itga8/Itgb1*.[73,86] In particular, Spp1 released by modulated SMCs as well as the disease-specific *Spp1*$^{+}$ fibroblast subset discussed previously governs the fibroblast and SMC functional changes that occur during TAA formation. In another study, ligand-receptor analyses revealed enhanced

collagen synthesis to be the most prominent feature of communication between fibroblasts and SMCs in Ang II-induced AAA mice, which was surprising considering that loss of collagen contributes to the development of AAA.[79] Interestingly, *Spp1-a9b1* comprised one of the ligand-receptor pairs either commonly significantly increased (*Spp1-a9b1*, *Fgfr1-Ncam1*) or decreased (*Nrp1-Vegfb*, *Pdgfr-Pdgfd*) in four expanded fibroblast clusters. Yang *et al.*[107] extensively inferred intercellular signaling from published scRNA-seq data of three experimental AAA models[70–72] as well as human AAA[82]. Important predictions include potential SPP1-mediated regulation of SMC phenotypic switching by inflammatory macrophages and altered thrombospondin (THBS) signaling in AAA.[107] This study also highlighted distinct differences between AAA mouse models, as exemplified by a contribution of vascular remodeling and fibrosis to early AAA progression in elastase- but not $CaCl_2$-induced AAA.

Other studies performed a more targeted analysis with a focus on specific interactions or ligand-receptor pairs. For instance, Dawson *et al.* performed junctional analyses of ligand-receptor pairs for *TGFB1-3* and bone morphogenic protein (BMP) ligands in human MFS and control tissues.[83] The highest TGFβ junctional scores were found between activated fibroblasts and all EC types, whereas BMP-mediated communication primarily occurred from de-differentiated ECs to all other non-immune cells. Another study estimated cell-cell and cell-ECM junction scores in ATAA patients and control subjects using the expression product method and found that modulated SMCs (fibromyocytes) exhibit higher junction scores, especially cell-ECM, than other SMC subsets.[81] The results of their analysis with regard to ECs and fibroblasts are discussed above. Cao *et al.* did comprehensively analyze the intercellular communication between immune cells and immune-related SMCs in this dataset, resulting in multiple important findings.[84] First, macrophages and macrophage-like SMCs showed the highest communication capabilities and acted as both signaling senders and receivers. Second, macrophage migration inhibitory factor (MIF) (*MIF-CD74*) and galectin (*LGALS9-CD44*) signaling stimulated SMC trans-differentiation into a macrophage-like phenotype, whereas C-X-C motif chemokine ligand (CXCL) (*CXCL12-CXCR4*) and galectin promoted SMC switching to a T-cell-like phenotype. Finally, the aforementioned signaling pathways together with complement (*C3-C3AR1*) and chemerin (*RARRES2-CMKLR1*) significantly contributed to AA progression by driving immune cell activation and migration. In agreement with the first finding, Wang *et al.* report that macrophages were the most notable signal senders and receivers in both TAA patients and controls, and together with ECs, fibroblasts, and SMCs comprised the most frequently interacting cells.[85] In TAA, their interactions with ECs and fibroblasts were significantly enhanced, whereas those with SMCs were reduced.

Conclusion and Future Perspectives

This review set out to shed light on the contribution that single-cell transcriptomics has made to expanding our knowledge of atherosclerosis and aortic aneurysm. Summarizing scRNA-seq findings has highlighted several remarkable similarities between these vascular pathologies. One striking parallel is an established immune component that is characterized in part by an accumulation of macrophages, some of which exhibit enhanced pro-inflammatory signaling (*Il1b*/*IL1B*, *TNF*). An increase in T cells is another shared feature, and comparable T-cell subsets include cytotoxic $CD8^+$ T cells (*Gzma*/*GZMA*, *Gzmk*/*GZMK*, *Ccl5*/*CCL5*) and T_{regs} (*tnfrsf18*/*TNFRSF18*). Additionally, EC dysfunction may further promote vascular inflammation in both atherosclerosis and AA by facilitating leukocyte recruitment (*Vcam1*/*VCAM1*). Another shared hallmark, as confirmed by trajectory inference, is the phenotypic modulation of SMCs. This phenomenon is often accompanied by an increase in ECM regulation (*Col1a1*/*COL1A1*, *Mgp*/*MGP*). With regard to cellular communication, shared frequently interacting cell types include fibroblasts, ECs, SMCs, and macrophages. Finally, complement signaling is an overlapping pathway that promotes immune cell activation and migration.

Nonetheless, our review also underscores differences between these vascular diseases. Notably, fibroblasts have been more prominently described in AA pathogenesis, whereas in atherosclerosis the immune component has a somewhat more pronounced role. There are also marked dissimilarities in immune cell subsets. For instance, foamy macrophages are a hallmark component of atherosclerotic lesions but were not particularly detected in AA tissues. Although, a $Trem2^+$ osteoclast-like macrophage has been described in AA as well. Instead, AA-associated macrophages contribute to ECM degradation and tissue remodeling by secreting MMPs. Similarly, some T-cell populations are seemingly unique to either atherosclerosis or AA, such as multilineage-committed $CD4^+$ T cells and switched T cells, respectively. Furthermore, within AA a clear plasma cell population has been described, whereas in single-cell studies of atherosclerosis the B cell lineage is not yet fully characterized. A notable discrepancy with regard to SMC modulation is that no equivalent of the SEM intermediate cell state in atherosclerosis has (yet) been identified in AA. Moreover, SMC-derived macrophages are more commonly found in atherosclerotic lesions, while SMCs in AA often activate stress response pathways, consistent with the greater loss of SMCs during AA formation. Other dissimilarities in cellular transitions are that EndMT is specific for atherosclerosis, whereas the re-differentiation of macrophages and fibroblasts occurs predominantly in AA. Major differences in cellular crosstalk include greater communication by T cells and macrophages in atherosclerosis and AA, respectively. Lastly, we did not identify any shared signaling pathways other than complement, with *C3-C3AR1* being the only common ligand-receptor pair.

The scRNA-seq findings presented here have a number of therapeutic implications. First, the disease-enriched cell populations identified through scRNA-seq provide potential therapeutic targets. miR-33 silencing has already been shown to drive plaque regression by reducing inflammatory and Trem2 macrophages.[44] Similarly, the success of the CANTOS (Canakinumab Anti-Inflammatory Thrombosis Outcome Study) trial, which targeted interleukin (IL)-1β[114], may be attributable to its impact on pro-inflammatory macrophages. Conversely, stimulating the aggregation of pro-resolving cell subtypes, such as M2 macrophages, may promote vascular disease regression. The next steps should focus on further elucidating and verifying the specific regulatory mechanisms and functions of these cell populations, in particular at different disease stages. Second, scRNA-seq has uncovered transcriptome factors involved in regulating SMC identity, such as *KLF4*.[37] Therapeutic strategies targeting such factors could manipulate SMC fates, halting disease progression or promoting its reversal. This applies not only to SMC phenotypic switching but also to other pathological transitions, such as EndMT. Of note, another potential approach for interfering with EndMT is to target two of its drivers as confirmed by scRNA-seq, i.e., arterial flow and stiffness. Further research should validate the factors that govern cellular plasticity and elucidate the mechanisms by which it contributes to disease development. Lastly, cellular communication profiling has revealed novel signaling pathways and ligand-receptor pairs that are active in vascular disease and hence consist of potential druggable targets. Examples include the cytokines amphiregulin and Spp1, which are specific for respectively atherosclerosis and AA, and the complement and CXCL signaling pathways, which seemingly contribute to both vascular pathologies. This knowledge provides a basis for therapeutic avenues that may disrupt this cellular crosstalk. However, further investigation is required to enhance our understanding of how this cell-cell communication drives vascular dysfunction and disease development. In particular, intervention studies should clarify whether the aforementioned shared signaling pathways play similar roles in atherosclerosis and AA. Additionally, drug-gene interaction analysis may facilitate the translation of scRNA-seq findings into new therapies by revealing candidate drug-gene interactions.[10]

It is essential for the findings that support novel therapeutic strategies to be corroborated by other studies. However, matching outcomes from different studies is currently being impeded by the considerable variation in transcriptome-based names of cell populations across studies.[58] This is illustrated by the variety of terms used for describing modulated SMCs. Hence, the field would benefit greatly from a unified nomenclature of cell populations. Future research should therefore focus on integrating the publicly available scRNA-seq data, especially for AA, since

meta-analyses in this field are currently lacking. This would not only help gain a comprehensive understanding of current studies but may also to some extent eliminate technical variance among studies, reveal novel cell subsets, and facilitate cross-species comparisons. Inter-species comparisons are of especially great importance considering that for many cell types it remains unclear how populations in mice and humans overlap. Lastly, a consensus in cell population nomenclature would simplify comparisons among vascular diseases.

Current limitations of scRNA-seq provide a multitude of opportunities for the future. One such limitation is the loss of spatial information, which has led to the emergence of spatial transcriptomics. This enables gene expression profiling across tissue space, allowing for pinpointing the location of specific cell populations in the vascular wall. It also helps decipher local networks of cell-cell communication by revealing ligand-receptor interactions between neighboring cells. Several techniques for spatial sequencing have been developed (reviewed in 115), including spatial barcoding and *in situ* hybridization. The application of these approaches in atherosclerosis and AA research would provide a more comprehensive functional landscape of these vascular pathologies. Still, no method is currently able to provide a full scope of the transcriptome at a single-cell resolution, highlighting the need for future advancements to make it an even more powerful tool.

Similarly, combining scRNA-seq with other single-cell omics approaches has the potential to greatly expand our knowledge of healthy and diseased vasculature. Such approaches may profile molecular features such as chromatin accessibility and histone modifications, providing insight into the epigenetic regulation of dynamic gene expression. Other single-cell omics assays measure the proteome or interactions between transcription factors and DNA. Two recent studies combined scRNA-seq with single-cell T-cell receptor sequencing (scTCR-seq) to map the T-cell repertoire in human coronary plaques[108] or human carotid lesions and matched peripheral blood mononuclear cell (PBMC) samples[116], finding evidence of clonal expansion. Future developments in integrative single-cell multi-omics technologies will allow us to measure multiple molecular profiles in the same cell, which will deepen our understanding of the complex biological processes that underlie atherosclerosis and AA development.

Another emerging trend that has the potential to provide profound information is the integration of scRNA-seq data with data from genome-wide association studies (GWASs). GWASs have uncovered numerous common genetic variants associated with vascular diseases, including coronary artery disease (CAD)[117]. However, translating

these genetic loci into biological mechanisms and candidate genes with clinical potential still poses a considerable challenge. To address this issue, three studies intersected GWAS summary statistics with scRNA-seq data of human plaques to map CAD susceptibility loci[50,118] or atherosclerotic and cardiovascular disease traits[51] to specific cell populations, thereby identifying potential atherosclerosis-associated gene-cell pairs. Thus, this approach represents a powerful tool for guiding future mechanistic research and discovering novel cell-specific drug targets for functional testing. Other (future) applications of scRNA-seq, such as understanding sex differences, the translation of observations from mice to men, and cardiovascular precision medicine, are discussed elsewhere.[119,120]

To conclude, single-cell transcriptomics has proven to be of tremendous value in the field of atherosclerosis and AA. On account of scRNA-seq, cell populations in the diseased vascular wall have been shown to be much more heterogeneous and dynamic than previously thought. Moreover, our understanding of cellular plasticity and cell-cell communication in disease pathogenesis has vastly improved. Still, the growing amount of scRNA-seq data amplifies the need for integrated analyses to unify cell population nomenclature and facilitate cross-species comparisons. Promising future opportunities lie in the combination of scRNA-seq with complementary technologies, such as spatial transcriptomics. We believe scRNA-seq will be instrumental in elucidating the molecular mechanisms underlying atherosclerosis and AA and in identifying candidate actionable pathways for drug discovery.

References

1. Roth, R., Kim, S., Kim, J. & Rhee, S. Single-cell and spatial transcriptomics approaches of cardiovascular development and disease. *BMB Rep* **53**, 393–399 (2020).
2. Tsao, C. W. *et al.* Heart Disease and Stroke Statistics-2022 Update: A Report From the American Heart Association. *Circulation* **145**, E153–E639 (2022).
3. Libby, P. *et al.* Atherosclerosis. *Nat Rev Dis Primers* **5**, 1–18 (2019).
4. Sakalihasan, N. *et al.* Abdominal aortic aneurysms. *Nat Rev Dis Primers* **4**, 1–22 (2018).
5. Senser, E. M., Misra, S. & Henkin, S. Thoracic Aortic Aneurysm: A Clinical Review. *Cardiol Clin* **39**, 505–515 (2021).
6. Tang, F. *et al.* mRNA-Seq whole-transcriptome analysis of a single cell. *Nat Methods* **6**, 377–382 (2009).
7. Nguyen, A., Khoo, W. H., Moran, I., Croucher, P. I. & Phan, T. G. Single cell RNA sequencing of rare immune cell populations. *Front Immunol* **9**, 1553 (2018).
8. Kester, L. & van Oudenaarden, A. Single-Cell Transcriptomics Meets Lineage Tracing. *Cell Stem Cell* **23**, 166–179 (2018).
9. Armingol, E., Officer, A., Harismendy, O. & Lewis, N. E. Deciphering cell–cell interactions and communication from gene expression. *Nat Rev Genet* **22**, 71–88 (2020).
10. Ma, W. F. *et al.* Enhanced single-cell RNA-seq workflow reveals coronary artery disease cellular cross-talk and candidate drug targets. *Atherosclerosis* **340**, 12–22 (2022).
11. Ma, W. F. *et al.* PlaqView 2.0: A comprehensive web portal for cardiovascular single-cell genomics. *Front Cardiovasc Med* **9**, (2022).
12. Yap, C., Mieremet, A., De Vries, C. J. M., Micha, D. & De Waard, V. Six Shades of Vascular Smooth Muscle Cells Illuminated by KLF4 (Krüppel-Like Factor 4). *Arterioscler Thromb Vasc Biol* **41**, 2693–2707 (2021).
13. Galkina, E. *et al.* Lymphocyte recruitment into the aortic wall before and during development of atherosclerosis is partially L-selectin dependent. *J Exp Med* **203**, 1273–1282 (2006).
14. Chavkin, N. W. & Hirschi, K. K. Single Cell Analysis in Vascular Biology. *Front Cardiovasc Med* **7**, 42 (2020).
15. Gu, W. *et al.* Adventitial Cell Atlas of wt (Wild Type) and ApoE (Apolipoprotein E)-Deficient Mice Defined by Single-Cell RNA Sequencing. *Arterioscler Thromb Vasc Biol* **39**, 1055–1071 (2019).
16. He, D. *et al.* Aortic heterogeneity across segments and under high fat/salt/glucose conditions at the single-cell level. *Natl Sci Rev* **7**, 881–896 (2020).
17. Hu, Z. *et al.* Single-Cell Transcriptomic Atlas of Different Human Cardiac Arteries Identifies Cell Types Associated With Vascular Physiology. *Arterioscler Thromb Vasc Biol* **41**, 1408–1427 (2021).
18. Kan, H. *et al.* Single-cell transcriptome analysis reveals cellular heterogeneity in the ascending aortas of normal and high-fat diet-fed mice. *Exp Mol Med* **53**, 1379–1389 (2021).
19. Yu, L. *et al.* An intersegmental single-cell profile reveals aortic heterogeneity and identifies a novel Malat1+ vascular smooth muscle subtype involved in abdominal aortic aneurysm formation. *Signal Transduct Target Ther* **7**, 1–14 (2022).
20. Kalluri, A. S. *et al.* Single-Cell Analysis of the Normal Mouse Aorta Reveals Functionally Distinct Endothelial Cell Populations. *Circulation* **140**, 147–163 (2019).
21. Lukowski, S. W. *et al.* Single-Cell Transcriptional Profiling of Aortic Endothelium Identifies a Hierarchy from Endovascular Progenitors to Differentiated Cells. *Cell Rep* **27**, 2748-2758.e3 (2019).

22. Jambusaria, A. *et al.* Endothelial heterogeneity across distinct vascular beds during homeostasis and inflammation. *Elife* **9**, (2020).
23. Kalucka, J. *et al.* Single-Cell Transcriptome Atlas of Murine Endothelial Cells. *Cell* **180**, 764-779. e20 (2020).
24. Gao, Y. K. *et al.* A regulator of G protein signaling 5 marked subpopulation of vascular smooth muscle cells is lost during vascular disease. *PLoS One* **17**, (2022).
25. Dobnikar, L. *et al.* Disease-relevant transcriptional signatures identified in individual smooth muscle cells from healthy mouse vessels. *Nat Commun* **9**, 1–17 (2018).
26. Tang, J. *et al.* Arterial Sca1+ Vascular Stem Cells Generate De Novo Smooth Muscle for Artery Repair and Regeneration. *Cell Stem Cell* **26**, 81-96.e4 (2020).
27. Weinberger, T. *et al.* Ontogeny of arterial macrophages defines their functions in homeostasis and inflammation. *Nat Commun* **11**, 1–16 (2020).
28. Cochain, C. *et al.* Single-Cell RNA-Seq Reveals the Transcriptional Landscape and Heterogeneity of Aortic Macrophages in Murine Atherosclerosis. *Circ Res* **122**, 1661–1674 (2018).
29. Jones, R. C. *et al.* The Tabula Sapiens: A multiple-organ, single-cell transcriptomic atlas of humans. *Science (1979)* **376**, (2022).
30. Elmentaite, R., Domínguez Conde, C., Yang, L. & Teichmann, S. A. Single-cell atlases: shared and tissue-specific cell types across human organs. *Nature Reviews Genetics 2022 23:7* **23**, 395–410 (2022).
31. Winkels, H. *et al.* Atlas of the Immune Cell Repertoire in Mouse Atherosclerosis Defined by Single-Cell RNA-Sequencing and Mass Cytometry. *Circ Res* **122**, 1675–1688 (2018).
32. Kim, K. *et al.* Transcriptome analysis reveals nonfoamy rather than foamy plaque macrophages are proinflammatory in atherosclerotic murine models. *Circ Res* **123**, 1127–1142 (2018).
33. Wirka, R. C. *et al.* Atheroprotective roles of smooth muscle cell phenotypic modulation and the TCF21 disease gene as revealed by single-cell analysis. *Nat Med* **25**, 1280–1289 (2019).
34. Lin, J. Da *et al.* Single-cell analysis of fate-mapped macrophages reveals heterogeneity, including stem-like properties, during atherosclerosis progression and regression. *JCI Insight* **4**, (2019).
35. Pan, H. *et al.* Single-Cell Genomics Reveals a Novel Cell State During Smooth Muscle Cell Phenotypic Switching and Potential Therapeutic Targets for Atherosclerosis in Mouse and Human. *Circulation* **142**, 2060–2075 (2020).
36. Williams, J. W. *et al.* Limited proliferation capacity of aortic intima resident macrophages requires monocyte recruitment for atherosclerotic plaque progression. *Nat Immunol* **21**, 1194–1204 (2020).
37. Alencar, G. F. *et al.* Stem Cell Pluripotency Genes Klf4 and Oct4 Regulate Complex SMC Phenotypic Changes Critical in Late-Stage Atherosclerotic Lesion Pathogenesis. *Circulation* **142**, 2045–2059 (2020).
38. Vafadarnejad, E. *et al.* Dynamics of Cardiac Neutrophil Diversity in Murine Myocardial Infarction. *Circ Res* **127**, E232–E249 (2020).
39. Wang, Y. *et al.* Clonally expanding smooth muscle cells promote atherosclerosis by escaping efferocytosis and activating the complement cascade. *Proceedings of the National Academy of Sciences* **117**, 15818–15826 (2020).
40. Kim, J. B. *et al.* Environment-sensing aryl hydrocarbon receptor inhibits the chondrogenic fate of modulated smooth muscle cells in atherosclerotic lesions. *Circulation* **142**, 575–590 (2020).
41. Wolf, D. *et al.* Pathogenic Autoimmunity in Atherosclerosis Evolves From Initially Protective Apolipoprotein B100-Reactive CD4+ T-Regulatory Cells. *Circulation* **142**, 1279–1293 (2020).
42. Sharma, M. *et al.* Regulatory T Cells License Macrophage Pro-Resolving Functions During Atherosclerosis Regression. *Circ Res* **127**, 335–353 (2020).

43. Schlegel, M. *et al.* Silencing Myeloid Netrin-1 Induces Inflammation Resolution and Plaque Regression. *Circ Res* **129**, 530–546 (2021).
44. Afonso, M. S. *et al.* miR-33 Silencing Reprograms the Immune Cell Landscape in Atherosclerotic Plaques. *Circ Res* **128**, 1122–1138 (2021).
45. Burger, F. *et al.* Single-Cell RNA-Seq Reveals a Crosstalk between Hyaluronan Receptor LYVE-1-Expressing Macrophages and Vascular Smooth Muscle Cells. *Cells* **11**, (2022).
46. Brandt, K. J. *et al.* Single-Cell Analysis Uncovers Osteoblast Factor Growth Differentiation Factor 10 as Mediator of Vascular Smooth Muscle Cell Phenotypic Modulation Associated with Plaque Rupture in Human Carotid Artery Disease. *Int J Mol Sci* **23**, (2022).
47. Chattopadhyay, A. *et al.* Preventing Cholesterol-Induced Perk (Protein Kinase RNA-Like Endoplasmic Reticulum Kinase) Signaling in Smooth Muscle Cells Blocks Atherosclerotic Plaque Formation. *Arterioscler Thromb Vasc Biol* **42**, 1005–1022 (2022).
48. Yao, F. *et al.* Histone Variant H2A.Z Is Required for the Maintenance of Smooth Muscle Cell Identity as Revealed by Single-Cell Transcriptomics. *Circulation* **138**, 2274–2288 (2018).
49. Fernandez, D. M. *et al.* Single-cell immune landscape of human atherosclerotic plaques. *Nat Med* **25**, 1576–1588 (2019).
50. Depuydt, M. A. C. *et al.* Microanatomy of the Human Atherosclerotic Plaque by Single-Cell Transcriptomics. *Circ Res* **127**, 1437–1455 (2020).
51. Slenders, L. *et al.* Intersecting single-cell transcriptomics and genome-wide association studies identifies crucial cell populations and candidate genes for atherosclerosis. *European Heart Journal Open* **2**, (2021).
52. Alsaigh, T., Evans, D., Frankel, D. & Torkamani, A. Decoding the transcriptome of calcified atherosclerotic plaque at single-cell resolution. *Commun Biol* **5**, 1–17 (2022).
53. Biswas, I., Khan, G. A., Biswas, I. & Khan, G. A. Endothelial Dysfunction in Cardiovascular Diseases. *Basic and Clinical Understanding of Microcirculation* (2019) doi:10.5772/INTECHOPEN.89365.
54. Souilhol, C., Harmsen, M. C., Evans, P. C. & Krenning, G. Endothelial-mesenchymal transition in atherosclerosis. *Cardiovasc Res* **114**, 565–577 (2018).
55. Bennett, M. R., Sinha, S. & Owens, G. K. Vascular Smooth Muscle Cells in Atherosclerosis. *Circ Res* **118**, 692–702 (2016).
56. Gomez, D. & Owens, G. K. Smooth muscle cell phenotypic switching in atherosclerosis. *Cardiovasc Res* **95**, 156–164 (2012).
57. Holmes, C. & Stanford, W. L. Concise review: stem cell antigen-1: expression, function, and enigma. *Stem Cells* **25**, 1339–1347 (2007).
58. Conklin, A. C. *et al.* Meta-Analysis of Smooth Muscle Lineage Transcriptomes in Atherosclerosis and Their Relationships to In Vitro Models. *Immunometabolism* **3**, (2021).
59. Zhang, Z., Huang, J., Wang, Y. & Shen, W. Transcriptome analysis revealed a two-step transformation of vascular smooth muscle cells to macrophage-like cells. *Atherosclerosis* **346**, 26–35 (2022).
60. Zernecke, A. *et al.* Meta-analysis of leukocyte diversity in atherosclerotic mouse aortas. *Circ Res* **127**, 402–426 (2020).
61. Cochain, C., Saliba, A. E. & Zernecke, A. Letter by Cochain et al regarding article, "Transcriptome analysis reveals nonfoamy rather than foamy plaque macrophages are proinflammatory in atherosclerotic murine models". *Circ Res* **123**, E49–E48 (2018).
62. Kim, K. & Choi, J. H. Response by Kim and Choi to Letter Regarding Article, 'Transcriptome Analysis Reveals Nonfoamy Rather Than Foamy Plaque Macrophages Are Proinflammatory in Atherosclerotic Murine Models'. *Circ Res* **123**, E50 (2018).

63. Zernecke, A. *et al.* Integrated single-cell analysis based classification of vascular mononuclear phagocytes in mouse and human atherosclerosis. *Cardiovasc Res* (2022) doi:10.1093/CVR/CVAC161.
64. Jonasson, L., Holm, J., Skalli, O., Bondjers, G. & Hansson, G. K. Regional Accumulations of T Cells, Macrophages, and Smooth Muscle Cells in the Human Atherosclerotic Plaque. *Arteriosclerosis* **6**, 131–138 (1986).
65. Hansson, G. K. & Jonasson, L. The discovery of cellular immunity in the atherosclerotic plaque. *Arterioscler Thromb Vasc Biol* **29**, 1714–1717 (2009).
66. Winkels, H. & Wolf, D. Heterogeneity of T Cells in Atherosclerosis Defined by Single-Cell RNA-Sequencing and Cytometry by Time of Flight. *Arterioscler Thromb Vasc Biol* **41**, 549–563 (2021).
67. Butcher, M. J. *et al.* Atherosclerosis-Driven Treg Plasticity Results in Formation of a Dysfunctional Subset of Plastic IFNγ+ Th1/Tregs. *Circ Res* **119**, 1190–1203 (2016).
68. Srikakulapu, P. *et al.* Artery Tertiary Lymphoid Organs Control Multilayered Territorialized Atherosclerosis B-Cell Responses in Aged ApoE-/- Mice. *Arterioscler Thromb Vasc Biol* **36**, 1174–1185 (2016).
69. Silvestre-Roig, C. *et al.* Externalized histone H4 orchestrates chronic inflammation by inducing lytic cell death. *Nature* **569**, 236–240 (2019).
70. Hadi, T. *et al.* Macrophage-derived netrin-1 promotes abdominal aortic aneurysm formation by activating MMP3 in vascular smooth muscle cells. *Nat Commun* **9**, 1–16 (2018).
71. Yang, H., Zhou, T., Stranz, A., Deroo, E. & Liu, B. Single-Cell RNA Sequencing Reveals Heterogeneity of Vascular Cells in Early Stage Murine Abdominal Aortic Aneurysm-Brief Report. *Arterioscler Thromb Vasc Biol* **41**, 1158–1166 (2021).
72. Zhao, G. *et al.* Single-cell RNA sequencing reveals the cellular heterogeneity of aneurysmal infrarenal abdominal aorta. *Cardiovasc Res* **117**, 1402–1416 (2021).
73. Pedroza, A. J. *et al.* Single Cell Transcriptomic Profiling of Vascular Smooth Muscle Cell Phenotype Modulation in Marfan Syndrome Aortic Aneurysm. *Arterioscler Thromb Vasc Biol* **40**, 2195 (2020).
74. Luo, W. *et al.* Critical Role of Cytosolic DNA and Its Sensing Adaptor STING in Aortic Degeneration, Dissection, and Rupture. *Circulation* **141**, 42–66 (2020).
75. Chen, P. Y. *et al.* Smooth Muscle Cell Reprogramming in Aortic Aneurysms. *Cell Stem Cell* **26**, 557 (2020).
76. Li, B. *et al.* Single-Cell Transcriptome Profiles Reveal Fibrocytes as Potential Targets of Cell Therapies for Abdominal Aortic Aneurysm. *Front Cardiovasc Med* **8**, 1635 (2021).
77. Sawada, H. *et al.* Second Heart Field-Derived Cells Contribute to Angiotensin II-Mediated Ascending Aortopathies. *Circulation* **145**, 987–1001 (2022).
78. Liu, X. *et al.* Single-cell RNA sequencing identifies an Il1rn+/Trem1+ macrophage subpopulation as a cellular target for mitigating the progression of thoracic aortic aneurysm and dissection. *Cell Discov* **8**, 1–21 (2022).
79. Weng, Y. *et al.* Single-Cell RNA Sequencing Technology Revealed the Pivotal Role of Fibroblast Heterogeneity in Angiotensin II-Induced Abdominal Aortic Aneurysms. *DNA Cell Biol* **41**, 498–520 (2022).
80. Pedroza, A. J. *et al.* Embryologic Origin Influences Smooth Muscle Cell Phenotypic Modulation Signatures in Murine Marfan Syndrome Aortic Aneurysm. *Arterioscler Thromb Vasc Biol* **42**, 1154–1168 (2022).

81. Li, Y. *et al.* Single-Cell Transcriptome Analysis Reveals Dynamic Cell Populations and Differential Gene Expression Patterns in Control and Aneurysmal Human Aortic Tissue. *Circulation* **142**, 1374–1388 (2020).
82. Davis, F. M. *et al.* Inhibition of macrophage histone demethylase JMJD3 protects against abdominal aortic aneurysms. *J Exp Med* **218**, (2021).
83. Dawson, A. *et al.* Single-Cell Analysis of Aneurysmal Aortic Tissue in Patients with Marfan Syndrome Reveals Dysfunctional TGF-beta Signaling. *Genes (Basel)* **13**, 95 (2021).
84. Cao, G. *et al.* Deciphering the Intercellular Communication Between Immune Cells and Altered Vascular Smooth Muscle Cell Phenotypes in Aortic Aneurysm From Single-Cell Transcriptome Data. *Front Cardiovasc Med* **9**, (2022).
85. Wang, Q. *et al.* Integrating Bulk Transcriptome and Single-Cell RNA Sequencing Data Reveals the Landscape of the Immune Microenvironment in Thoracic Aortic Aneurysms. *Front Cardiovasc Med* **9**, (2022).
86. Zhou, M. *et al.* Fibroblast-Secreted Phosphoprotein 1 Mediates Extracellular Matrix Deposition and Inhibits Smooth Muscle Cell Contractility in Marfan Syndrome Aortic Aneurysm. *J Cardiovasc Transl Res* (2022) doi:10.1007/S12265-022-10239-8.
87. Cheng, S. *et al.* Identification of key monocytes/macrophages related gene set of the early-stage abdominal aortic aneurysm by integrated bioinformatics analysis and experimental validation. *Front Cardiovasc Med* **9**, (2022).
88. Folkersen, L. *et al.* Unraveling divergent gene expression profiles in bicuspid and tricuspid aortic valve patients with thoracic aortic dilatation: the ASAP study. *Molecular medicine* **17**, 1365–1373 (2011).
89. Chen, C., Liao, Y. & Peng, G. Connecting past and present: single-cell lineage tracing. *Protein Cell* **13**, 790 (2022).
90. Wagner, D. E. & Klein, A. M. Lineage tracing meets single-cell omics: opportunities and challenges. *Nat Rev Genet* **21**, 410–427 (2020).
91. Shankman, L. S. *et al.* KLF4-dependent phenotypic modulation of smooth muscle cells has a key role in atherosclerotic plaque pathogenesis. *Nat Med* **21**, 628–637 (2015).
92. Gaddis, D. E. *et al.* Apolipoprotein AI prevents regulatory to follicular helper T cell switching during atherosclerosis. *Nat Commun* **9**, 1–15 (2018).
93. Deng, J. *et al.* Single-cell gene profiling and lineage tracing analyses revealed novel mechanisms of endothelial repair by progenitors. *Cellular and Molecular Life Sciences* **77**, 5299–5320 (2020).
94. Saelens, W., Cannoodt, R., Todorov, H. & Saeys, Y. A comparison of single-cell trajectory inference methods. *Nat Biotechnol* **37**, 547–554 (2019).
95. Trapnell, C. *et al.* The dynamics and regulators of cell fate decisions are revealed by pseudotemporal ordering of single cells. *Nat Biotechnol* **32**, 381–386 (2014).
96. Qiu, X. *et al.* Reversed graph embedding resolves complex single-cell trajectories. *Nat Methods* **14**, 979–982 (2017).
97. La Manno, G. *et al.* RNA velocity of single cells. *Nature* **560**, 494–498 (2018).
98. Tritschler, S. *et al.* Concepts and limitations for learning developmental trajectories from single cell genomics. *Development (Cambridge)* **146**, (2019).
99. Andueza, A. *et al.* Endothelial Reprogramming by Disturbed Flow Revealed by Single-Cell RNA and Chromatin Accessibility Study. *Cell Rep* **33**, (2020).
100. Li, F. *et al.* Single-cell RNA-seq reveals cellular heterogeneity of mouse carotid artery under disturbed flow. *Cell Death Discov* **7**, (2021).

101. Zamani, M. *et al.* Single-Cell Transcriptomic Census of Endothelial Changes Induced by Matrix Stiffness and the Association with Atherosclerosis. *Adv Funct Mater* 2203069 (2022) doi:10.1002/ADFM.202203069.
102. Von Rossum, A., Laher, I. & Choy, J. C. Immune-mediated vascular injury and dysfunction in transplant arteriosclerosis. *Front Immunol* **5**, (2015).
103. Ramilowski, J. A. *et al.* A draft network of ligand–receptor-mediated multicellular signalling in human. *Nat Commun* **6**, 1–12 (2015).
104. Almet, A. A., Cang, Z., Jin, S. & Nie, Q. The landscape of cell-cell communication through single-cell transcriptomics. *Curr Opin Syst Biol* **26**, 12–23 (2021).
105. Efremova, M., Vento-Tormo, M., Teichmann, S. A. & Vento-Tormo, R. CellPhoneDB: inferring cell–cell communication from combined expression of multi-subunit ligand–receptor complexes. *Nat Protoc* **15**, 1484–1506 (2020).
106. Jin, S. *et al.* Inference and analysis of cell-cell communication using CellChat. *Nat Commun* **12**, 1–20 (2021).
107. Yang, H., DeRoo, E., Zhou, T. & Liu, B. Deciphering Cell-Cell Communication in Abdominal Aortic Aneurysm From Single-Cell RNA Transcriptomic Data. *Front Cardiovasc Med* **9**, 831789 (2022).
108. Chowdhury, R. R. *et al.* Human Coronary Plaque T Cells Are Clonal and Cross-React to Virus and Self. *Circ Res* **130**, 1510–1530 (2022).
109. Browaeys, R., Saelens, W. & Saeys, Y. NicheNet: modeling intercellular communication by linking ligands to target genes. *Nat Methods* **17**, 159–162 (2019).
110. Giladi, A. *et al.* Dissecting cellular crosstalk by sequencing physically interacting cells. *Nat Biotechnol* **38**, 629–637 (2020).
111. Ximerakis, M. *et al.* Single-cell transcriptomic profiling of the aging mouse brain. *Nat Neurosci* **22**, 1696–1708 (2019).
112. Farbehi, N. *et al.* Single-cell expression profiling reveals dynamic flux of cardiac stromal, vascular and immune cells in health and injury. *Elife* **8**, (2019).
113. Kumar, M. P. *et al.* Analysis of Single-Cell RNA-Seq Identifies Cell-Cell Communication Associated with Tumor Characteristics. *Cell Rep* **25**, 1458-1468.e4 (2018).
114. Ridker, P. M. *et al.* Antiinflammatory Therapy with Canakinumab for Atherosclerotic Disease. *N Engl J Med* **377**, 1119–1131 (2017).
115. Longo, S. K., Guo, M. G., Ji, A. L. & Khavari, P. A. Integrating single-cell and spatial transcriptomics to elucidate intercellular tissue dynamics. *Nat Rev Genet* **22**, 627–644 (2021).
116. Depuydt, M. A. C. *et al.* Single-cell T cell receptor sequencing of paired human atherosclerotic plaques and blood reveals autoimmune-like features of expanded effector T cells. *Nature Cardiovascular Research 2023 2:2* **2**, 112–125 (2023).
117. Aragam, K. G. *et al.* Discovery and systematic characterization of risk variants and genes for coronary artery disease in over a million participants. *Nat Genet* **54**, 1803–1815 (2022).
118. Örd, T. *et al.* Single-Cell Epigenomics and Functional Fine-Mapping of Atherosclerosis GWAS Loci. *Circ Res* **129**, 240–258 (2021).
119. Slenders, L., Tessels, D. E., Laan, S. W. van der, Pasterkamp, G. & Mokry, M. The Applications of Single-Cell RNA Sequencing in Atherosclerotic Disease. *Front Cardiovasc Med* **9**, 826103 (2022).
120. Fernandez, D. M. & Giannarelli, C. Immune cell profiling in atherosclerosis: role in research and precision medicine. *Nat Rev Cardiol* **19**, 43–58 (2022).