Hypertension

REVIEW

Aortic Cellular Heterogeneity in Health and Disease: Novel Insights Into Aortic Diseases From Single-Cell RNA Transcriptomic Data Sets

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ABSTRACT: Aortic diseases such as atherosclerosis, aortic aneurysms, and aortic stiffening are significant complications that can have significant impact on end-stage cardiovascular disease. With limited pharmacological therapeutic strategies that target the structural changes in the aorta, surgical intervention remains the only option for some patients with these diseases. Although there have been significant contributions to our understanding of the cellular architecture of the diseased aorta, particularly in the context of atherosclerosis, furthering our insight into the cellular drivers of disease is required. The major cell types of the aorta are well defined; however, the advent of single-cell RNA sequencing provides unrivaled insights into the cellular heterogeneity of each aortic cell type and the inferred biological processes associated with each cell in health and disease. This review discusses previous concepts that have now been enhanced with recent advances made by single-cell RNA sequencing with a focus on aortic cellular heterogeneity.

Key Words: aortic diseases ■ cardiovascular diseases ■ collagen ■ extracellular matrix proteins ■ hypertension

Cardiovascular disease is the leading cause of death, with chronic hypertension being the primary cause.[1] Hypertension places significant stress on the aorta, promoting aortic pathologies such as atherosclerosis, aortic aneurysms, and aortic stiffening.[2] Upregulation of proinflammatory, profibrotic, and hypertrophic pathways is strongly associated with structural and functional changes within the vessel wall.[1] These processes ultimately lead to damage in major organs such as the heart, kidneys, and brain.[1] In this review, we highlight analogous cellular mechanisms in each of the major aortic cell types in different aortic diseases. We also discuss recent advances made by single-cell RNA sequencing (scRNA-seq), focusing on aortic cellular heterogeneity in health and disease.

AORTIC STRUCTURE

The aorta is the largest artery in the body and responsible for transporting oxygenated blood from the heart to the systemic circulation.[3] Like all arteries, the aorta is comprised of 4 layers.[3] From the lumen outwards these are the: tunica intima (a monolayer of endothelial cells; ECs), tunica media (predominantly vascular smooth muscle cells; VSMCs), tunica adventitia (fibroblasts and leukocytes), and perivascular adipose tissue (PVAT; adipocytes, leukocytes, fibroblasts).[3] ECM (extracellular matrix) proteins are present in each of these layers.[4] The function and structure of the aorta varies along its length which is referred to as regional heterogeneity.[4] Because of this, the aorta is often separated into

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Supplemental Material is available at https://www.ahajournals.org/doi/suppl/10.1161/HYPERTENSIONAHA.123.20597.
For Sources of Funding and Disclosures, see page 748.

Hypertension is available at www.ahajournals.org/journal/hyp

Nonstandard Abbreviations and Acronyms

AAA	abdominal aortic aneurysms
ACh	acetylcholine
ADMA	asymmetrical dimethylarginine
CCR2	CC chemokine receptor-2
EC	endothelial cell
ECM	extracellular matrix
eNOS	endothelial NO synthase
IFN-γ	interferon gamma
IL	interleukin
MMP	matrix metalloproteinase
PVAT	perivascular adipose tissue
ROS	reactive oxygen species
scRNA-seq	single-cell RNA sequencing
TNF-α	tumor necrosis factor-alpha
VSMC	vascular smooth muscle cell

4 regions: ascending, aortic arch, thoracic (descending), and abdominal (Figure 1).[4]

Blood flow and pressure also vary throughout the length of the aorta.[2] This allows for vital functions, such as absorbing high pressures during physiological (ie, exercise and pregnancy) and pathophysiological (ie, hypertension) stressors.[2] Although the ascending aorta and aortic arch are similar in composition, the adventitia changes considerably throughout the length of the descending aorta and is thicker in the abdominal region compared with the thoracic.[5] The most obvious regional heterogeneity is decreased medial thickness as distance from the heart increases.[6] ECM proteins also vary between the different regions.[6] Elastin content is highest in the aortic arch and decreases along the descending aorta.[6] In contrast, collagen content increases with distance from the heart (ranging from ≈22% of aortic wall content in the proximal ascending aorta to ≈53% in the abdominal aorta).[7] PVAT also varies throughout the regions, whereby brown adipocytes are abundant in the aortic arch and thoracic aorta.[8] Conversely, beige and white adipocytes are more prevalent in the descending aorta.[8]

AORTIC FUNCTION

The aorta is exposed to high pulse pressures and must be highly elastic and compliant to endure this.[3] Elastin provides aortic compliance, enabling the aorta to act as a reservoir to store part of the stroke volume during

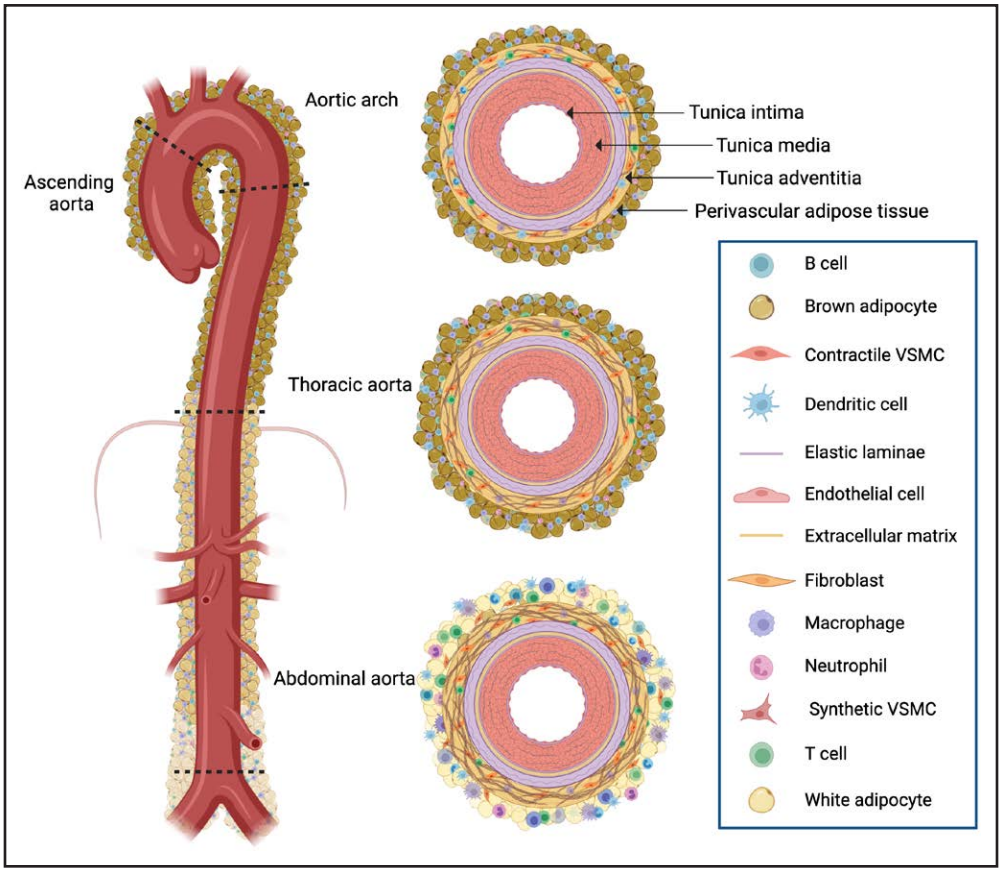

Figure 1. Regional heterogeneity of the healthy aorta.
The cellular composition varies between the ascending aorta, aortic arch, thoracic aorta, and abdominal aorta (dotted lines indicate the boundaries of each section). VSMC indicates vascular smooth muscle cell. Created with BioRender.com.

each systolic cycle.[3] This blood volume is later released from the aorta during each diastolic cycle.[4] This phenomenon is known as the Windkessel effect,[3] which reduces blood pressure waveform downstream, and is essential in maintaining aortic compliance throughout the entirety of the cardiac cycle.[4]

Collagen also plays a crucial role in the aorta by providing structural stability to withstand high luminal pressures.[9] Types I and III collagens account for ≈80% of all aortic collagen.[9] Finally, aortic tone (defined as the level of constriction of the aortic wall), which is primarily mediated by the endothelium and VSMCs, is another crucial regulator of blood flow.[10] Aortic constriction increases resistance which decreases blood flow to downstream smaller arteries.[10] This in turn increases pulse wave velocity which inhibits the Windkessel effect.[3] This can further exacerbate the stress and strain on the vessel wall to promote vascular diseases such as aortic stiffening (characterized by increased aortic collagen) or hypertension.[3] Although aortic stiffening does occur during aging,[11] this process is accelerated in many disease states.

AORTIC DISEASE

There are a range of aortic diseases that contribute to poor aortic function (ie, hypertension, aneurysms, and atherosclerosis). Although each of these diseases have differing etiologies, there are common pathological mechanisms between these. These include inflammation, ECM remodeling, vascular dysfunction, and oxidative stress.[12] Pathophysiological changes of aortic structure and function can be both a cause and consequence of hypertension.[12] The main cell types contributing to these mechanisms include ECs,[13] VSMCs,[14] fibroblasts,[15] and leukocytes.[16–18] These cell types are often identified using common gene/protein markers (Tables S1 and S2 defines all genes cited in this article). Recent advances in scRNA-seq have allowed for in-depth understanding of the cellular heterogeneity of these key aortic cell types in healthy and disease states.

SINGLE-CELL RNA SEQUENCING

Single-cell technologies offer an unprecedented characterization of complex tissue types in health and disease. Unlike bulk RNA sequencing, which provides the average gene expression for a given population of cells, scRNA-seq offers high-throughput sequencing of each individual cell. This allows for the characterization of cellular heterogeneity and intercellular and intracellular communication pathways within a given tissue.[19] This is crucial for identifying novel cell types or mechanisms that may serve as new highly specific targets for aortic diseases.

Since its first use in 2009,[20] scRNA-seq has been applied to many different tissues and disease states, including the aorta (Table). Following tissue harvest, tissue digestion is the initial step in the scRNA-seq workflow, whereby a single-cell (or single nuclei) suspension is prepared.[42] Samples are sometimes then enriched for live cells or specific cell types using fluorescence-activated cell sorting. Single-cell suspensions then undergo single-cell isolation to identify each individual cell that is analyzed. Commonly used approaches include: microdroplet-based (ie, 10× Genomics and Drop-seq), micromanipulation (ie, CEL-seq and MATQ-seq), or FACS (MARs-seq and FLASH-seq).[43] RNA is then extracted from each cell, cDNA synthesized and amplified for library construction. Libraries are then sequenced and data are computationally analyzed using a reference genome that aligns to the sequenced cDNA.[19] Cells can then be identified in an unbiased fashion, annotated, and further interrogated using bioinformatic pipelines with emerging complexities[44] (Figure 2).

AORTIC CELLULAR HETEROGENEITY IN HEALTH AND DISEASE

Endothelial Cells

In the healthy aorta, ECs maintain aortic homeostasis and regulate vascular tone.[45] This occurs via the release of vasoactive factors that regulate VSMC contraction and relaxation.[45] Key aortic vasoactive factors are NO and prostacyclin for relaxation, and endothelin and vasoconstrictor prostanoids (such as thromboxane) for contraction.[45] Endothelial dysfunction is a well-defined hallmark of many aortic diseases and typically characterized by the reduced production of vasodilatory factors or increased production of vasoconstrictors.[13] This disrupts vascular tone to promote inflammation and leukocyte adhesion within the aortic wall.[13] Sustained endothelial dysfunction also increases endothelium permeability, platelet aggregation, and cytokine release—all of which promote vascular wall degradation and drive aortic diseases such as atherosclerosis and aneurysms.[13]

NO is a key EC-derived factor that affects vascular function and is generated by endothelial NO synthase (eNOS).[46] Genetic deletion of eNOS (*Nos3*$^{-/-}$), leads to vascular inflammation characterized by proinflammatory macrophages (ie, M1) and proinflammatory cytokines (ie, IL [interleukin]-6, TNF-α [tumor necrosis factor-alpha], IFN-γ [interferon gamma]).[47] This chronic inflammation exacerbates several aortic diseases such as: hypertension, atherosclerosis, aneurysms, and neointimal hyperplasia. Moreover, eNOS deficiency also increases blood pressure,[48] and *Nos3*$^{-/-}$ mice are a commonly used model of hypertension.

Despite this clear role of eNOS in maintaining vascular function, most scRNA-seq studies have not identified changes in *Nos3* in diseased or damaged EC populations.

Table. Summary of Aortic Single-Cell RNA Sequencing Data Sets to Date

Species	Cells in data set	Reads/cell	No. of subclusters					Myofibroblasts identified	Reference
			Total	EC	VSMC	Fibro	Leukocyte		
Healthy conditions									
Mouse	≈6200	17 000	10	3	1	2	1 Mac; 2 Mon	Y	21
Aging									
Mouse	28 014	N/A	16	2	1	1	1 DC; 1 Mac/Mon; 2 T cell; 1 B cell	N	22
Hypertension									
Mouse	36 076	N/A	19	3	3	3	1 Mac; 1 Mon; 3 T cell; 1 NK; 2 B cell	N	23
Rat	≈21 000	≈140 000	15	6	9	10	1 DC; 4 Mac; 1 Mon; 1 T cell	N	24
Aneurysms									
Human	48 128	N/A	40	2	3	5	1 DC; 8 Mac; 1 Mon; 11 T cell; 1 NK; 1 B cell	Y	25
Human	52 337	≈50 000	26	1	4	9	3 Mac; 3 Mon; 3 T cell; 2 NK; 1 B cell	N	26
Mouse	5000	N/A	8	2	2	3	1 Mac	N	27
Mouse	≈1500	≈289 000	17	1	4	2	1 DC; 5 Mac/Mon; 1 T cell; 1 B cell	N	28
Mouse	3896	>50 000	12	1	2	2	1 DC; 3 Mac; 1 T cell/NK; 1 B cell	N	29
Mouse	61 826	N/A	23	1	13	4	3 Mac; 1 T cell; 1 B cell	Y	30
Mouse	5424	≈285 000	15	1	1	4	4 Mac/Mon; 3 T cell; 3 B cell	N	31
Mouse	18 835	N/A	15	3	8	2	1 DC; 2 Mac/ Mono; 2 T cell/NK; 1 B cell	N	32
Mouse	>25 000	≈34 000	24	3	2	10	1 DC; 6 Mac/Mon; 1 T cell; 1 B cell	Y	33
Mouse	17 252	≈50 000	10	1	1	3	4 Mac; 1 T cell; 2 NK; 1 B cell	N	34
Mouse	26 257	60, 470	8	2	6	2	1 Mac; 1 Mon; 1 T cell	N	35
Mouse	13 427	N/A	8	4	3	1	0	N	36
Atherosclerosis									
Mouse	2986	257 000	11	0	0	0	1 DC; 3 Mac; 6 T cell/NK; 2 B cell	N	37
Mouse	1059	10 million	12	0	0	0	4 Mac; 2 Mon/DC; 4 T cell; 1 NK; 1 B cell	N	38
Mouse	2054	>150, 000	5	2	1	1	1 Mac	N	39
Obesity									
Mouse	24 001	N/A	27	3	5	4	1 DC; 4 Mac/Mon; 1 T cell; 3 B cell	N	40
Mouse	216 612	118 608	28	3	3	4	1 DC; 1 Mac; 4 T cell; 2 B cell	N	41

AAA indicates abdominal aortic aneurysm; DC; dendritic cell; NK; EC, endothelial cell; Fibro, fibroblast; Mac, macrophages; Mon, monocytes; NA; not available; NK, natural killer cells; and VSMC; vascular smooth muscle cell.

This may be due to the posttranscriptional phosphorylation of eNOS that regulates NO production.[49] Thus, changes in eNOS function may not correlate with changes in *Nos3* gene expression. Ultimately, this may mean that scRNA-seq is not suitable for assessing NO signaling. However, in 1 scRNA-seq study *Nos3* was enriched in activated ECs in the mouse aorta.[41] This population was reported as a classical EC—characterized by the unique expression of *Nos3* and enriched for genes that contribute to the vascular tone regulation (ie, *Edn1*, *Ace*). This was further validated using immunofluorescence.[41] Activated ECs were also enriched for vessel dilation and blood pressure regulation gene pathways.[41] These gene pathways differed significantly from those enriched in other EC subtypes, where processes such as inflammatory response to wound healing,[23] proteoglycan expression,[41] and ECM organization[21] were enriched. Although *Nos3* expression was unchanged in disease, activated EC abundance was reduced in unhealthy conditions (ie, mice fed a high-salt, high-fat, or high-sugar diet).

Various scRNA-seq data sets have consistently identified ≈2 to 3 distinct EC subtypes (Table), highlighting the heterogeneity of aortic EC. EC populations were consistently identified based on high *Cdh5*[21,40] or *Pecam1* expression.[22] However, some studies used additional markers unique to certain EC subsets (including *Vwf* and *Vcam1*—which have been confirmed using immunofluorescence studies).[21,22,40] In data sets with a third EC subcluster, these were typically identified as a lymphatic ECs based on *Lyve1* expression.[21,40]

Most studies indicate that different EC subclusters line the aorta, with the abundance of each subcluster varying between aortic regions.[21,22,41] Moreover, EC function changes significantly in response to various diseases of the aorta (ie, high-fat diet, aneurysm, and hypertension). In healthy conditions, subclusters were typically enriched for angiogenesis, proliferation, migration, and endothelial barrier establishment.[21] Conversely, wound healing, inflammatory responses and collagen genes including *Col4a3, Col5a2*, and *Col8a1* were enriched in

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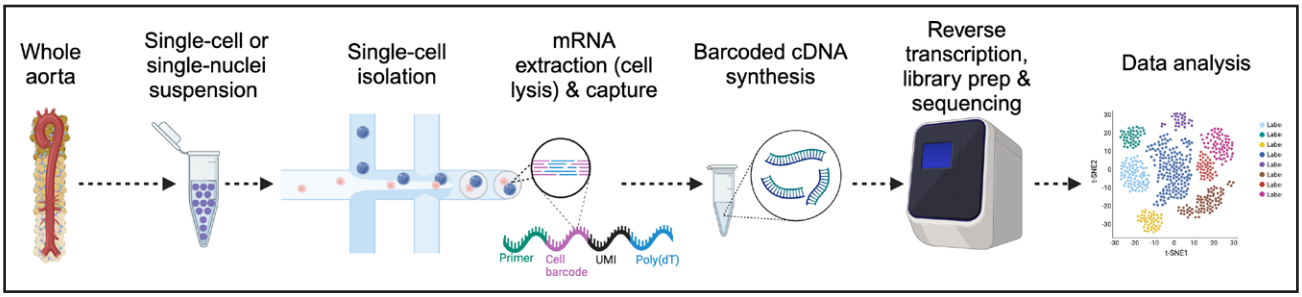

Figure 2. Workflow of single-cell and single nuclei-RNA sequencing.
An overview of single cell and single nuclei-RNA sequencing workflow on samples from whole aorta. Samples are prepared into either a single cell or single nuclei suspension and single cells are isolated (droplet microfluidics approach pictured). mRNA is extracted from lysed cells, cDNA is synthesized and amplified via reverse transcription. Gene libraries are constructed, and samples are then sequenced. The final step of single cell or single nuclei-sequencing is to perform downstream analyses. Created with BioRender.com.

EC subclusters in disease.[21,23,41] However, scRNA-seq data sets have reported variable EC function in disease states. For example, while total aortic EC abundance did not change in high-fat diet-fed mice, the abundance of a specific subcluster enriched for biological processes associated with cell proliferation, body fluids regulation, and vascular development was increased.[40] High-fat diet feeding also increased the expression calcium-binding protein S100a8 in this subcluster. This molecule regulates the immune response and inflammation.[40] Similarly, in salt-induced hypertension only 1 aortic EC subcluster increased in abundance—likely due to proliferation. Gene expression of reactive oxygen species (ROS)-related enzymes, collagen, contractility, and histocompatibility complex genes were all upregulated in hypertension in this EC subcluster.[23] In the context of aging, scRNA-seq identified 2 aortic EC subclusters with similar biological processes (ie, vasculature development and EC proliferation); however, nitrite analyses revealed that NO production significantly varied between each EC subcluster. The abundance of the low NO-producing subcluster also increased with aging.[22]

Although most studies are yet to validate the functions of distinct EC subclusters in vivo or in vitro, scRNA-seq data sets further support the notion for endothelial dysfunction in aortic disease. Collectively, these data highlight that inflammation and ECM remodeling are the major cellular mechanisms enriched in aortic ECs in disease. Interestingly, reduced EC vasodilatory function has not been detected using scRNA-seq. Thus, it is unclear whether these newly identified cellular mechanisms precede or cause vasodilatory deficits in aortic disease. Moreover, studies are yet to explore the feasibility of targeting disease-specific endothelial subclusters to treat endothelial dysfunction.

Vascular Smooth Muscle Cells

Aortic VSMCs control vascular tone through contraction and relaxation in response to both intrinsic and extrinsic (predominantly endothelial) vasoactive factors.[50] VSMCs are surrounded by ECM, primarily elastin with some collagen to offer both flexibility and stability to the aortic wall, respectively. These ECM components are organized into elastic laminae.[51] VSMCs are typically classified as contractile or synthetic—which describes their primary function.[52] Contractile and synthetic VSMCs have been identified using both conventional (ie, immunohistochemistry and cell culture) and scRNA-seq studies.[22,25,53] Contractile VSMCs are more abundant than synthetic VSMCs in healthy conditions[14,54] and characterized by higher expression of contractile markers (ie, *Acta2*, *Tagln*, *Smthn*, *Myh11*, and *Cnn1*).[55]

Most scRNA-seq studies identify multiple VSMC subclusters[24,28,30] (Table), including those with either a contractile or synthetic phenotype.[23,40] In a study of elastase-induced abdominal aortic aneurysms (AAA), 4 VSMC subclusters were identified and were defined as quiescent contractile, proliferative contractile, differentiated, and proinflammatory subclusters. No synthetic phenotype was detected,[28] but the differentiated VSMC subcluster was enriched for *Klf4*,[28] a known driver of VSMC switching to a synthetic phenotype.[56] This suggests the differentiated VSMC subcluster was contractile VSMCs switching to a synthetic phenotype.

Increased synthetic VSMC abundance is a well-recognized characteristic of aortic disease.[40,57] Contractile VSMC markers are typically downregulated in synthetic VSMCs, while ECM remodeling genes (ie, *Spp1*, *Mmp9*, *Ereg*, and *Vim*) are enriched.[58–60] This not only exacerbates ECM production, but also reduces contractility.[57] Historically, synthetic VSMC markers are thought to be somewhat contentious,[61] thus, many studies use cellular morphology to identify synthetic VSMCs.[62] Typically, synthetic VSMCs have an irregular/rhomboid morphology, whereas contractile VSMCs are spindled.[62]

One of the first steps in aortic disease is VSMC switching from a contractile to a synthetic phenotype.[63] Although the precise mechanisms of this are largely unknown, many stimuli drive this phenomenon (ie, inflammation, ROS, sheer and mechanical stress, Ang [angiotensin] II, and aldosterone).[64] Chronic exposure to these

stimuli increases MMP (matrix metalloproteinase) and ECM protein (including collagen, fibrillin, and proteoglycans) expression in synthetic VSMCs.[65] It remains unknown if synthetic VSMCs are reparative or detrimental in disease. For example, synthetic VSMCs promote aortic wall repair via migration, proliferation, and ECM protein synthesis.[66] However, synthetic VSMCs also promote aortic stiffening, via VSMC hypertrophy, elastin breakdown, and collagen production.[67,68] Whether synthetic VSMCs are beneficial or detrimental may vary between disease states.

Fibroblasts

Fibroblasts are the primary mediators of aortic remodeling.[69] Although fibroblasts are essential for ECM synthesis and homeostasis, the role of aortic fibroblasts heavily depends on their phenotype.[69] For example, resident fibroblasts in a quiescent state (nonproliferating or nonactivated), play an essential role in ECM homeostasis. Resident fibroblasts differentiate into activated fibroblasts in response to a range of stimuli (including Ang II, TGF-β, chronic inflammation, and mechanical/sheer stress) and in many aortic diseases (including hypertension, AAA, atherosclerosis, and vascular injury).[69–71] Activated fibroblasts promote disease development. This is typically in response to cytokines which can induce further release of cytokines (including TGF-β1,[72] IL-6, and MCP-1[73]) to promote inflammation and fibroblast proliferation.[74] Activated fibroblasts also produce excessive amounts of ECM proteins (including collagens, fibronectin, and proteoglycans).[74] TGF-β is considered the main cytokine that stimulates fibroblast activation and ECM protein production in diseased and injured tissues.[72] In the heart, this occurs primarily via the SMAD3 pathway,[75] which stimulates ECM production, leukocyte recruitment, and the differentiation of activated fibroblasts into a third phenotype, known as myofibroblasts.[76] It is unclear if this same mechanism drives myofibroblast differentiation in the aorta.

Myofibroblasts are classically identified by the coexpression of *Acta2* and the traditional fibroblast markers *Col1a1* and *Pdgfra*.[77] Myofibroblasts are typically upregulated in aortic diseases, and considered the primary source of pathological ECM deposition.[78,79] Although TGF-β is a well-characterized driver of aortic fibrosis and myofibroblast differentiation, in vitro studies suggest this also occurs via other stimuli such as Ang II, phosphor-ERK1/2,[80] and TNF-α.[81] Most of our knowledge of fibroblast function stems from in vitro techniques due to the lack of fibroblast-specific markers. Moreover, recent scRNA-seq studies reveal that fibroblasts are incredibly heterogeneous.

The distinct phenotype and specificity of myofibroblasts to aortic disease states, suggests that they may be a suitable target for new therapies that could halt or reverse aortic stiffening. However, previous studies that attempted to target myofibroblasts and activated fibroblasts suggest that this may not be possible. For example, ≈50% of TGF-β1-deficient mice die in utero due to insufficient and defective vasculogenesis.[82] Surviving mice demonstrated normal fetal growth, however, significant inflammation was present in many organs by 2 to 4 weeks of age, which was ultimately fatal.[82] Similarly, high mortality rates also occurred in TGF-β2-deficient mice and TGF-β3-deficient pups.[83,84]

Collectively, these data highlight the crucial role of TGF-β in early development. Despite TGF-β cytokines being expressed in many of cell types, no studies have specifically targeted these pathways in fibroblasts—highlighting that global gene knockout models may not be appropriate to study the role of TGF-β in aortic disease. One study explored the effect of a mouse monoclonal TGF-β IgG_1 antibody in a mouse model of Ang II-induced AAA.[85] TGF-β antibody-reduced serum TGF-β1 to negligible levels.[85] This was associated with increased ascending and suprarenal aortic aneurysm rupture, suggesting that TGF-β1 neutralization is detrimental in AAA, and that myofibroblasts may prevent AAA rupture during hypertensive conditions.[85] Overall, the high mortality and advanced disease progression associated with global TGF-β depletion, suggests that this is an inappropriate target for interrogating fibroblast function. Better insights could be gained from scRNA-seq studies, to guide the development of more specific treatments that do not interfere with essential fibroblast functions.

scRNA-seq data sets suggest that fibroblasts are highly heterogeneous in the aorta[23,31,33] (Table). Ten functionally distinct fibroblast populations were identified in a mouse model of Ang II-induced AAA.[33] Of these fibroblast subclusters, 4 increased in abundance in AAA mice, and 5 subclusters decreased in abundance.[33] Moreover, biological processes (ie, binding of collagen and the synthesis of ECM and degradation of ECM) and genes (ie, *Acta2*, *Fbln1*, and *Fn1*) related to fibrosis were enriched in fibroblasts from AAA mice.[33] Cell-to-cell communication analysis revealed that most ligand-receptor connections occurred between fibroblasts and VSMCs.[33] Collagen production was the main feature of these communications, suggesting that fibroblasts promote a synthetic VSMC phenotype during AAA formation.[33] Interestingly, no *Acta2* expression was detected in any of the fibroblast subclusters, suggesting that myofibroblasts do not exist in the healthy aortae or in AAAs.[33] Moreover, other studies using mouse models of AAA did not identify $Acta2^{+}$ fibroblasts or myofibroblasts.[27,28,34]

Another study analyzing the cellular landscape of the aorta using an apolipoprotein E deficient ($ApoE^{-/-}$) mouse AAA model identified only 3 aortic fibroblasts populations.[34] Unlike the previous study, a small population of $Acta2^{+}$ fibroblasts were detected.[34] Additionally, an $Acta2^{-}$ subcluster was classified as inactivated fibroblasts and an $Acta2^{low}$ subcluster as an intermediate

subtype.[34] Despite their distinct phenotypes, all 3 subclusters were present in the healthy and diseased aorta.[34] Moreover, AAA enriched the same biological processes in all 3 subclusters including extracellular structure organization and ECM organization.[34]

Although many murine studies did not detect *Acta2* expression in aortic fibroblasts, 1 human[25] and 2 murine studies[30,33] have identified *Acta2*$^{+}$ fibroblasts (Table). The lack of *Acta2* expression in aortic fibroblasts may be due to low sequencing depth. Interestingly, while *Acta2* expression was not always detected within fibroblast subclusters in these other disease states (Table), 1 study identified *Acta2*$^{+}$ myofibroblasts in the healthy mouse aorta.[21]

Collectively, these studies on aortic fibroblasts emphasize the essential balance between physiological and pathophysiological ECM deposition. The notion to target fibroblasts to prevent aortic disease is complex, as many of the mechanisms involved in aortic disease are crucial in physiological aortic remodeling. The high cellular resolution of scRNA-seq identifies some genes that are specific to the disease state (ie, *Spp1, Ccn2, Cfd*, and *Comp*).[33,35] Future studies should explore these to selectively target profibrotic fibroblast phenotypes, while allowing nonprofibrotic fibroblast populations to conduct physiological tissue repair.

Leukocytes

Chronic inflammation plays a critical role in aortic diseases.[86] Various stimuli promote leukocyte infiltration into the aortic wall.[87] These leukocytes then promote disease progression by the release of cytokines, ROS, and adhesion molecules.[87] Moreover, during aortic remodeling, other aortic cell types also produce proinflammatory cytokines to stimulate inflammation (including TGF-β1, IL-1β, IL-33, CXC, and CC chemokines[88] and ROS[86]). Numerous leukocyte types are implicated in the development of aortic diseases,[89] particularly antigen presenting cells (ie, macrophages and dendritic cells; DCs) and lymphocytes.[18,90] Aortic scRNA-seq studies highlight that each of these cell types are heterogenous with functionally distinct subtypes (Table). The abundance of certain aortic leukocyte subtypes also change substantially with health and disease.

Macrophages

Macrophage accumulation is a hallmark of many aortic diseases including hypertension,[90] atherosclerosis,[91] and aortic dissection.[92] Macrophages have traditionally been separated into 2 phenotypes: M1 (classically activated) or M2 (alternatively activated).[18] M1 macrophages are considered a proinflammatory phenotype, as they produce proinflammatory cytokines such as TNF and IL-1β, ROS, and nitrogen species.[18] Indeed, M1 macrophages are essential for AAA formation.[93] Moreover, macrophage recruitment, MMP9 expression and aneurysm formation are reduced in TNF-deficient mice.[93] Conversely, M2 macrophages typically express trophic factors including fibroblast growth factor, fibronectin, and TNF that promote ECM remodeling.[94] M2 macrophages are derived from proinflammatory monocytes.[18] Disease states such as hypertension are associated with proinflammatory monocyte accumulation within the aortic wall via CCR2 (CC chemokine receptor-2) activity.[18,95] Studies using a CC-motif receptor-2 antagonist (INCB334) in Ang II-induced hypertension suggest that proinflammatory monocytes promote detrimental aortic ECM remodeling.[18] Specifically, INCB334-reduced systolic blood pressure and prevented monocyte and M2 macrophage infiltration, collagen deposition, and elastin loss.[18] Although aortic M2 macrophages may be detrimental in hypertension, they are likely protective in AAA as they coordinate aneurysm repair following M1 macrophage infiltration.[96]

Although many studies have consistently identified M1 and M2 macrophage phenotypes, this is an outdated paradigm. M1 (proinflammatory) macrophages are typically identified by *Cxcl2* and *Cd14* expression, whereas M2 (resident-like) macrophages by *Csfr* and *Cd163* expression.[37] More recent studies show that >2 macrophage phenotypes exist.[97] This is further supported by scRNA-seq data sets that consistently report >2 macrophage subclusters.[24,28,40] In a mouse model of β-aminopropionitrile-induced thoracic aortic aneurysm and dissection, scRNA-seq identified 3 distinct macrophage populations including proinflammatory, resident-like and CD74^{high} antigen-presenting populations.[30] All 3 macrophage populations were localized to the aortic adventitia using in situ hybridization.[30] MMP (including *Mmp2* and *Mmp9*) expression was markedly increased in all macrophage subclusters in thoracic aneurysm and dissection, especially in the proinflammatory population.[30] Moreover, other MMPs were expressed exclusively in AAA macrophage clusters (*Mmp3*, *Mmp8*, *Mmp14*, *Mmp16*, and *Mmp25*).[30] This MMP expression in the proinflammatory population was accompanied by expression of *Il1b* and *Tnf*, suggesting the proinflammatory population was the most detrimental of the 3 subclusters.[30]

Moreover, in a mouse model of atherosclerosis (11 or 20 weeks of a high-fat diet in low-density lipoprotein receptor-deficient mice), macrophages were the most abundant leukocyte within the aorta.[37] Three macrophage populations were identified including resident-like, inflammatory, and *Trem2*^{hi} (the most significantly enriched gene in this previously undefined macrophage) subclusters.[37] Although resident macrophage gene expression was consistent between healthy and diseased mice, the remaining 2 macrophage populations, were almost exclusive to the atherosclerotic aorta.[37] Inflammatory macrophages were localized to both mouse and human

atherosclerotic plaques using an anti-IL-1β antibody, as *Il1b* was the top enriched gene in inflammatory macrophages.[37] Unsurprisingly, the top enriched gene ontology term for proinflammatory macrophages was inflammatory response.[37] These macrophages were enriched for proinflammatory genes including *Cxcl2*, *Ccl3*, *Ccl4*, *Tlr2*, and *Nlrp3* and nuclear factor-$_{K}$B inhibitor-related genes such as *Nfkbia*, *Nfkbiz*, *Nfkbid*, and *Ier3*.[37] This subcluster was also enriched for *Zpf36*, a posttranscriptional suppressor of TNF-α and nuclear factor-$_{K}$B.[37] Enriched *Zpf36* expression in a proinflammatory macrophage subcluster was surprising given *Zpf36* is thought to limit atherosclerosis severity.[98] Increased *Zpf36* expression may be a compensatory mechanism to limit macrophage activation in disease.[37]

Following induction of elastase-induced-infrarenal AAA, leukocytes were markedly increased compared with control mice.[28] Five macrophage populations were identified, including resident, proliferative, blood-derived macrophages, as well as M1- and M2-like subclusters.[28] Moreover, classical markers were expressed by all macrophages (including *Cd68*, *Cd14*, and *Adgre1*, *H2-Aα*, and *Fcgr1*). However, 1 subcluster also expressed vascular remodeling and aneurysm formation markers (*Mmp9*, *Ctsc*, *Ctsd*, and *Ctss*).[28] Resident macrophages expressed both pro- and anti-inflammatory markers (*Ccl2*, *Ccl3*, *Ccl7*, *Ccl12*, *Cxcl2*, *Il1b*, and *Il10*), suggesting this cluster contributes to the resolution of inflammation following AAA progression.[28] These data highlight that macrophages may be involved in a broader range of functions rather than just the innate inflammatory response during AAA disease progression and resolution.[28]

In addition to typical macrophage populations, a hybrid fibroblast/leukocyte cell type, termed the fibrocyte, has been identified in aortic diseases—including atherosclerosis[99] and hypertension-induced aortic fibrosis.[88] Fibrocytes are commonly identified based on the coexpression of CD34, CD45, vimentin, and type I collagen.[100] As they are not typically present with healthy tissue, fibrocytes are thought to migrate from the bone marrow to injured tissues.[100,101] Once at their target tissue, fibrocytes secrete chemokines, attracting more fibrocytes and leukocytes.[101] Due to their hybrid phenotype, fibrocytes also secrete ECM proteins including collagen and fibronectin.[100] Unlike the majority of aortic scRNA-seq studies, 1 scRNA-seq study identified a fibrocyte subcluster that was distinct from macrophages, in the setting of murine AAA.[34] Surprisingly, fibrocytes were also present in the healthy aorta. Fibrocyte abundance increased by >2-fold in AAA in both mice and humans,[34] suggesting that aortic fibrocytes may also differentiate from resident macrophages.

Flow cytometry validated the presence of fibrocytes in human AAA.[34] It is proposed that fibrocytes have a reparative role in AAA.[34] Adoptive transfer of green fluorescent protein-labelled fibrocytes in Ang II-treated mice attenuated AAA formation (decreased lesion diameter), elastin degradation, and ultimately reduced mortality.[34] However, in Ang II-induced hypertension, fibrocytes are thought to be detrimental and promote aortic fibrosis.[88] These findings highlight the dynamic chemotactic characteristics and marked collagen production by fibrocytes, regardless of whether their role is beneficial or detrimental to a particular disease.

Dendritic Cells

Like macrophages, DCs provide a vital link between innate and adaptive immunity.[102] These antigen presenting cells actively monitor the tissue microenvironment for damage- and pathogen-associated molecular patterns to alert other leukocytes to initiate an immune response.[102] DCs exist within the healthy aorta and mainly reside close to the endothelium. They are broadly classified into conventional DCs that are myeloid-derived, and plasmacytoid DCs, which can be both myeloid or lymphoid derived.[103] Both conventional and plasmacytoid DCs are involved in various cardiovascular diseases.[104] Specifically, plasmacytoid DCs accumulate within atherosclerotic plaques and increased expression of interferon-α stimulates cytotoxic T-cell activation.[104] Collectively, these processes increase overall inflammation leading to plaque destabilization.[105] DCs infiltrate the aortic wall during early atherosclerosis development.[106] This infiltration is typically accompanied by local aortic T-cell proliferation.[106]

Selective deletion of MHC-II in plasmacytoid DCs reduced early atherosclerotic plaque development compared with control counterparts.[107] This protective effect was due to reduced T-cell infiltration within plaques.[107] Moreover, *ApoE*$^{-/-}$ mice that received continuous treatment of anti-mouse plasmacytoid DCs antigen 1 antibodies (selectively depleting plasmacytoid DCs in the aorta), also reduced atherosclerosis development.[108] This was associated with reduced aortic macrophages and increased plaque collagen content, suggesting plaque stabilization.[108] Overall, these data suggest that plasmacytoid DCs are detrimental in atherosclerosis.[108]

DCs also contribute to the development of Ang-II and deoxycorticosterone acetate-induced hypertension in mice.[109] Specifically, the B7 ligand CD86 in DCs was significantly increased in hypertension, resulting in T-cell accumulation and activation.[109] Moreover, genetic ablation of B7 ligands prevented hypertension and pharmacological blockade of the B7 ligand/CD28 T-cell axis reversed established hypertension.[109] This axis is the costimulatory interaction between CD28 and B7 ligands CD80 and CD86 to activate T cells. It is thought to be essential for the development of hypertension. Although multiple DCs phenotypes have been well characterized using single-cell techniques such as flow cytometry, aortic scRNA-seq data sets often identify only 1 DCs cluster.[22,40,41] The lack of heterogeneity may be due to

multiple factors including depth of sequencing, digestion protocol, or that DCs have not been target cells of interest and thus in-depth analysis has not occurred. Population size impacts heterogeneity in scRNA-seq analyses. The low abundance of aortic DCs is a key limitation for studying their heterogeneity aortic diseases using scRNA-seq. Future studies may address this by performing scRNA-seq on aortic cell preparations that are enriched for DCs.

T Cells

The adaptive immune system also contributes to aortic disease development. T cells mature into T helper/T regulatory ($CD4^+$) or cytotoxic ($CD8^+$) or double negative ($CD4^-$/CD8) T cells.[110] T cells are activated by T-cell receptor (TCR) binding to MHC molecules presented by APCs.[111] Following stimulation (or costimulation from TCR and MHC molecules) $CD4^+$ T cells differentiate into either T helper (Th) 1, Th2, Th17 cells, or T-regulator (Treg) cells, with each producing specific cytokines and functions.[112] $CD4^+$ T cells are strong mediators of aortic disease.[113] Specifically, $CD4^+$ T-cell depletion reduces aneurysm formation.[113] Administration of the T-cell-derived cytokine IFN-γ partially reversed this effect, highlighting the active role IFN-γ likely plays in aneurysm formation.[113] IFN-γ deficiency reduces MMP expression and aortic wall destruction in calcium chloride-induced AAA.[113] Moreover, *Rag1*$^{-/-}$ mice lacking T cells and B cells are protected from Ang II-induced hypertension.[16] Adoptive transfer of purified T but not B cells restores hypertension and vascular dysfunction,[16] suggesting that T cells are essential for the development of hypertension.

Interestingly, the types of aortic T-cell subclusters identified via scRNA-seq vary between disease states (Table). One scRNAseq study identified 5 T-cell subclusters ($CD4^+$/$CD8^+$, memory, Th17, Th2, $CD8^+$) in the atherosclerotic aorta.[114] Other studies identified only 2 aortic T-cell populations ($CD4^+$ and $CD8^+$) in aged mice,[22] and 3 distinct populations in mice with salt-sensitive hypertension.[23] Although the total abundance of Th17 and Th2 cells increased in aged mice, the proportion of $CD8^+$ T cells remained relatively unchanged.[23] In hypertensive mice, cytokine expression was enriched in T-cell subclusters (compared with normotensive controls), suggesting these cells were more proinflammatory.[23] Interestingly, only 1 aortic T-cell cluster was identified in spontaneously hypertensive rats—likely due to the small population size.[24] Nevertheless, aortic T cells increased in abundance by ≈3-fold in spontaneously hypertensive rats compared with control WKY rats.[24] Immunofluorescence revealed that T cells accumulated in both the endothelium and adventitia,[24] rather than the adventitia and PVAT, as previously reported in hypertensive mice.[16] The variability in T-cell heterogeneity in different scRNA-seq data sets suggests that T-cell phenotypes and differentiation may be disease-specific. However, differences in sample collection and preparation can impact on the populations identified within a data set.

B Cells

B cells are characterized by their antibody secreting ability and are well-recognized players in several aortic diseases (including Ang II-induced hypertension[17] and atherosclerosis[115]). B cells originate in the bone marrow and differentiate into a B1 or B2 lineage.[38] B1 cells mature into short-lived plasma cells (typically in the pleura and peritoneum), whereas B2 cells differentiate into transitional B cells (typically in the spleen).[38] Transitional B cells then differentiate into a naive mature B cells, further differentiating into mature, activated, memory, follicular, marginal zone, or plasma B cells.[116,117]

Genetic or pharmacological B cell depletion in mice highlights a crucial role for B cells in aortic disease. For example, mice deficient in the B cell-activating receptor lack mature B cells.[17] These mice are protected from Ang II-induced increases in serum immunoglobulin (IgG) and systolic blood pressure, aortic stiffening, and collagen deposition.[17] This was associated with marked reductions in aortic macrophages and TGF-β.[17] Moreover, adoptive transfer of B cells into B-cell-deficient mice restored hypertension.[17] In AAA, pharmacological B-cell depletion (via monoclonal antimouse CD20 antibody) suppressed AAA growth, despite comparable circulating IgG, and cytokine levels between placebo and B-cell-deficient mice.[118] B-cell depletion increased aortic Treg cells, suggesting that B cells regulate the T-cell response in AAA.[118]

Many techniques studying B cells are restricted by the number of markers that can be used per sample (ie, flow cytometry, immunohistochemistry).[119] This is because many B-cell subset phenotypes are similar, sometimes only being differentiated by the expression levels of the same markers, making it difficult to accurately define subtypes.[119] Although scRNA-seq has overcome this limitation in other cell types (ie, fibroblasts), the study of aortic B cells using scRNA-seq is still limited.

Most aortic scRNA-seq data sets identify only 1 aortic B-cell cluster.[22,28,29] However, 3 B-cell populations were identified in the aortic adventitia of *ApoE*$^{-/-}$ mice[31] and in the aorta of high-fat diet-fed mice.[40] However, neither of these studies focused on B cells, and thus none of the B-cell subsets were characterized. This lack of aortic B-cell subset identification in aortic scRNA-seq data sets is a major gap in the literature. Thus, current scRNA-seq studies have not provided many insights into B-cell biology in aorta.

LIMITATIONS AND FUTURE DIRECTIONS

The advancement of single-cell technologies has unequivocally revolutionized our understanding of aortic heterogeneity. However, limitations exist, and there are

cell types where scRNA-seq has only provided small increments of new knowledge (ie, VSMCs, B cells). The main challenge of scRNA-seq is the complexity of data produced. The high-throughput data also increases the risk of false-positive findings. Thus, researchers should be careful to not overinterpret results. Other limitations including sample preparation, bias in the reporting of findings and types of analyses performed.

Aortic digestions and single-cell suspension preparations are often study-specific, with no established standard method for the field. Consequently, the cellular composition and quality of sequenced samples can vary considerably. This is especially true when comparing data sets from different studies analyzing the same disease state (Table), that is, some studies identify many distinct fibroblast populations,[33] whereas others do not.[22,36,39] Moreover, quick and accurate sample processing is a major challenge of scRNA-seq, whereby RNA degradation can significantly impair downstream results. To reduce the likelihood of false-positive findings, DoubletFinder, a detection tool that can be incorporated into pipelines to remove technical artifacts, known as doublets," whereby a droplet contains >1 cell.[120] Although there have been advancements in sequencing technology, doublets are not always removed. This detection tool relies on gene expression to distinguish real doublets from singlets based on high portion of genes in a close proximity to 1 another.[120] Importantly, this pipeline limits false positives within a given data set.[120]

Another major variable between studies is sequencing depth (Table). A sequencing depth of 10 000 to 50 000 reads/cell is required to achieve unbiased cell-type classification within a heterogenous tissue.[121] Increasing sequencing depth increases mRNA reads, thus, increasing the number of genes detected per cell. Deeper sequencing allows for more detailed cell-cell communication and biological process analyses, often increasing cellular heterogeneity within the data sets.[122] However, 1 study compared low- and high-depth sequencing of healthy aortic tissue (17 000 versus 145 000 reads/cell) and reported no differences in cellular heterogeneity.[21]

Large scRNA-seq data sets often contain a lot of background noise." Quality control steps are vital for filtering out this noise during data processing—particularly that of low-quality cells (including broken, dead, or contaminated). If not removed properly, this background noise creates artefacts and greatly impairs downstream analyses. A big focus during quality control is placed on detecting high or low reads of mitochondrial RNA, which can indicate if a cell is broken or dying.[123] Clustering of scRNA-seq data sets can also be manipulated manually by the user, introducing bias. A possible approach to combat this, is to use unsupervised clustering where cells are clustered together based on gene expression similarity.[124]

One limitation of scRNA-seq particularly relevant to the aorta is the inability to sequence whole adipocytes, which are often too large or fragile for the required microfluidics. Thus, most aortic scRNA-seq data sets do not include adipocytes, despite growing recognition of their involvement in aortic health and disease.[125] Moreover, PVAT is sometimes removed.[21,23,41] This also removes other major cell types within the PVAT (ie, leukocytes and fibroblasts).

One approach that allows for the sequencing of large or fragile cell types is (snRNA-seq. snRNA-seq is an alternative technique that allows RNA to be read from the nuclei of each individual cell and removes the complexity of preparation of large and fragile cell types.[126] The workflow for snRNA-seq is similar to that of scRNA-seq; however, nuclei are isolated rather than individual cells (Figure 2).[126] An additional benefit to snRNA-seq is the minimal mitochondrial contamination that can often occupy scRNA-seq data sets. This is particularly beneficial as more reads are dedicated to nuclear genes rather than mitochondrial genes.[126] To our knowledge, only 2 studies to date have used this approach, analyzing human thoracic aortic aneurysms[127] and vascular aging in mice.[22] Although the human study identified a small population of adipocytes,[127] no adipocytes were identified in the mouse study. The authors concluded the lack of adipocytes may be due to their floating characteristic.[22] Two other relatively recent advancements in single-cell sequencing technologies that do not require the dissociation of tissue are spatial transcriptomics and high-plex in situ hybridization. These technologies perform cell-specific gene expression analysis while preserving spatial data. This allows for the precise visualization of where gene expression occurs, based upon classical microscopic techniques to capture genetic and spatial heterogeneity within a tissue. This also allows for the analysis of fragile cell types that would otherwise not endure the cell dissociation process.

CONCLUSIONS

Aortic diseases are multicellular processes, and scRNA-seq has immensely expanded our knowledge into the heterogeneity and complexity of the aorta. This has ultimately provided a deeper understanding of the cell types present within the healthy aorta, and how they change—both phenotypically and in abundance—in diseased states. Both scRNA-seq and snRNA-seq technologies provide unprecedented insights into the cellular mechanisms attributing to disease progression but are not suited for all cellular mechanisms. Vasoactive mechanisms are less suited for these technologies, when compared with leukocyte or ECM remodeling mechanisms. Importantly, all scRNA-seq or snRNA-seq findings are at the gene level, and thus require to validation at the protein level. Moving forward, employing spatially resolved

techniques such as high-plex in situ hybridization or spatial transcriptomics allows for the analysis of high-throughput transcriptomics data, while preserving spatial information. Spatially resolved data would also benefit from being combined with scRNA-seq data, to ensure consistent and in-depth sequencing is achieved. Overall, single-cell transcriptomics technologies are constantly advancing and may be our greatest tool in identifying much-needed new targets for treating aortic diseases.

ARTICLE INFORMATION

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Sources of Funding

T.A. Gibson Hughes was funded by an Australian Research Training Scholarship. M. Jelinic was funded by a joint National Medical Research Council of Australia and National Heart Foundation Early Career Fellowship (GNT1146314 and 101943, respectively).

Disclosures

None.

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