



# Cardiac fibroblast activation during myocardial infarction wound healing



## Fibroblast polarization after MI

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## Abstract

Cardiac wound healing after myocardial infarction (MI) evolves from pro-inflammatory to anti-inflammatory to reparative responses, and the cardiac fibroblast is a central player during the entire transition. The fibroblast mirrors changes seen in the left ventricle infarct by undergoing a continuum of polarization phenotypes that follow pro-inflammatory, anti-inflammatory, and pro-scar producing profiles. The development of each phenotype transition is contingent upon the MI environment into which the fibroblast enters. In this mini-review, we summarize our current knowledge regarding cardiac fibroblast activation during MI and highlight key areas where gaps remain.

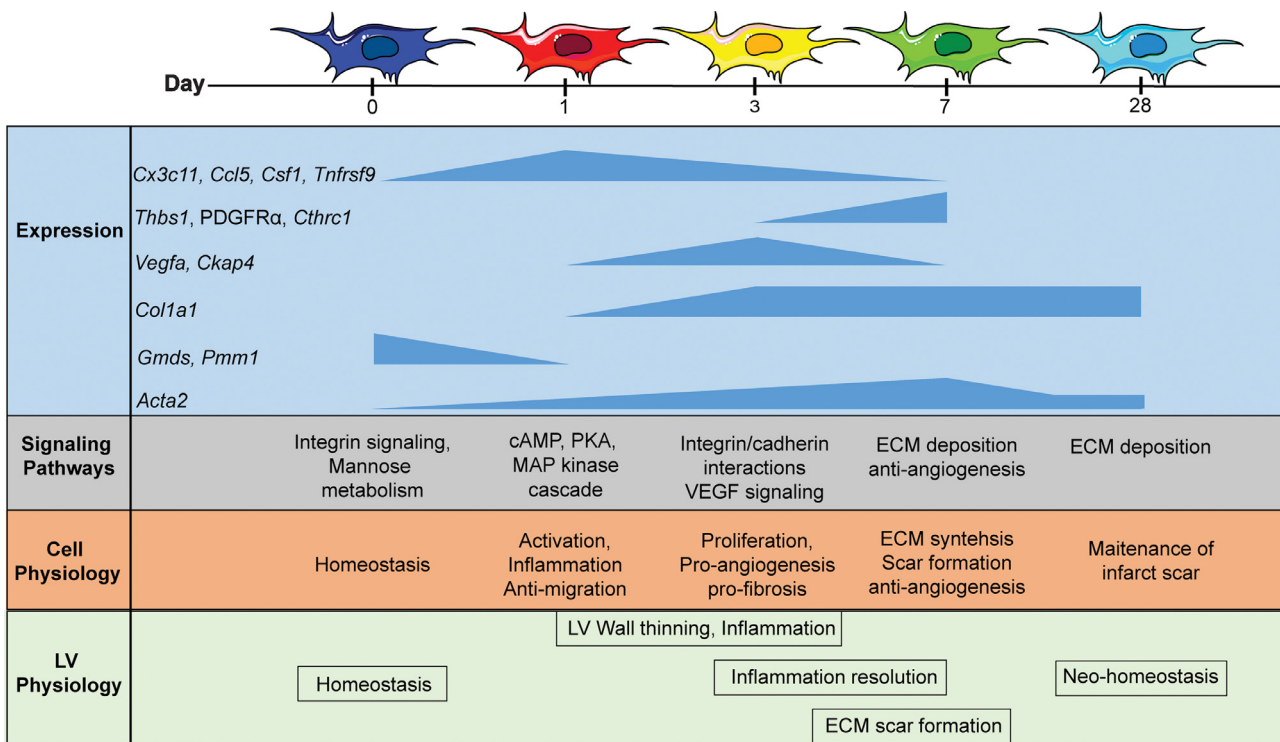
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## Introduction

Following myocardial infarction (MI), the left ventricle (LV) undergoes a series of wound healing responses that starts with an inflammatory reaction to clear necrotic myocytes and ends with the formation of an infarct scar composed of extracellular matrix (ECM). The infarct scar is dynamic and highly metabolically active, being comprised of cells and vasculature [1]. Long term outcomes are dependent on the quantity and quality of scar formed, as too little or too weak of a scar can generate LV aneurysm, and too much or too stiff of a scar can generate a rigid LV [2,3]. Too much ECM in the infarct scar can also serve as a conduit for arrhythmias. Therefore, a balance in ECM deposition and cross-linking is needed to minimize infarct expansion while maintaining an optimal tension. Because cardiac

fibroblasts are the major source of ECM in the MI LV, fibroblast responses determine both short and long term outcomes. The purpose of this mini-review is to provide a summary of recent findings made regarding the role of the cardiac fibroblast in the MI LV and detail the changes in fibroblast phenotype over the different phases of cardiac remodeling after MI (Fig. 1).

**Terminology, cell origins, and tissue culture considerations.** Fibroblast is a general term that encapsulates cells in the connective tissue that synthesize collagen and other ECM components [4]. While the term myofibroblast has been used to differentiate a fibroblast cell type that is  $\alpha$  smooth muscle actin (*Acta2*) positive, this terminology is limiting because it does not take into account the non-binary accumulation of *Acta2* occurs in fibroblasts of the MI LV and does not take into account other indices of



**Fig. 1. Cardiac fibroblast activation along the time continuum of response to myocardial infarction (MI).** Before MI, fibroblasts are homeostatic and producing extracellular matrix (ECM) to allow normal physiology. At MI day 1, fibroblasts convert to a pro-inflammatory phenotype, signaling activation of inflammatory signaling. At MI day 3, fibroblasts polarize to a proliferative and pro-angiogenesis profile. Peak expression in the genes listed correspond to adaptations in cell and left ventricle physiology (function). At MI day 7, fibroblasts promote ECM synthesis to form the infarct scar and inhibit angiogenic signaling. At MI day 28, fibroblasts return to a state of neohomeostasis, once again supporting the normal turnover of ECM to support the new infarct environment. Updated from [4].

cell activation [4-6]. Fibroblast activation is defined as the transition from a homeostatic fibroblast phenotype over the MI phases of cardiac remodeling. This is a broad term used to generically refer to any increase or change in fibroblast activity. Similar to other cardiac cell types such as macrophages and neutrophils [7,8], fibroblast activation in the setting of MI involves polarization to specific shifts in phenotype. Fibroblast activation or polarization cannot be easily assigned based on a single gene or protein marker, as transcriptomics and proteomics examinations have revealed a variety of fibroblast subtype populations. The number of subtypes in part reflect a variety of origins, and cardiac fibroblast origins shift over the MI time course. Fibroblasts from naïve uninjured myocardium reflect for the most part epicardial cells that underwent epithelial-to-mesenchymal transition during development [9]. After MI, cardiac fibroblasts originate from several sources, including resident cardiac fibroblasts, bone marrow derived progenitors, epicardial cells, and endothelial cells [10].

In tissue culture, cardiac fibroblasts respond to a vast array of stimuli that includes cytokines, growth factors, and hormones [1]. Although in vitro

stimulation of naïve fibroblasts provide information on how cells respond to single stimuli challenge, most in vivo responses represent a merging of similar and conflicting cues. Examining cardiac fibroblasts ex vivo actually provides the best means to understand its role in the context of MI. We have shown that cardiac fibroblasts retain their in vivo phenotype through passage 4 [5,10-14]. Two recent articles highlight the ex vivo memory of the MI fibroblast, as the Molkenin lab used freshly isolated fibroblasts and our team used cells to passage 3 for our evaluations [10,15]. The reproducibility between the two studies was excellent, revealing that isolating fibroblasts and culturing them on plastic in 10% fetal bovine serum provides a means to expand this cell while maintaining in vivo phenotypes. Attempts to recapitulate the perfect in vivo environment, therefore, are not necessary for ex vivo cardiac fibroblast assessments.

**Day 0 fibroblasts are homeostatic, continually sensing their environment to provide replacement ECM to maintain normal LV structure and physiology.** In normal LV, cardiac fibroblasts are a major non-cardiomyocyte cell type [16]. Previously, fibroblasts were thought to be quiescent or inactive,

and become activated in response to inflammation or other wounding signals. We now know that resident fibroblasts are continually assessing homeostasis in the environment. Enrichment analysis of gene expression for isolated Day 0 no MI fibroblasts from the LV shows high expression for genes involved in integrin signaling and glycolysis. These processes allow fibroblasts to respond to the surrounding environment and replace ECM as needed [17]. In particular, GDP-Mannose 4,6-Dehydratase (*Gmcs*) and Phosphomannomutase 1 (*Pmm1*) regulate synthesis of substrates in N-glycan biosynthesis, which includes a number of ECM components, and both genes are downregulated with MI [10]. Expression of these genes allows fibroblasts to maintain basal ECM production. Fibroblasts in the uninjured myocardium respond to stretch and are constantly monitoring the environment during the contraction and relaxation cycle [15,18]. Fibroblast activation induces suppression of sarcolemmal K(ATP) channel opener P-1075 inducing depolarization of the myocardium [19]. While fibroblasts are not excitable, they interact with cardiomyocytes in part through shared expression of connexins 40, 43, and 45 to aid in transduction of electrical potentials throughout the myocardium [19,20]. Resident cardiac fibroblasts, therefore, have primarily homeostatic roles in the uninjured myocardium to preserve ECM production and assist the cardiomyocyte in providing normal contractile function.

**Day 1 MI fibroblasts are pro-inflammatory and anti-migratory.** MI initiates an inflammatory phase of LV remodeling. Necrotic cardiomyocytes release damage associated molecular patterns such as high motility group box 1 (HMGB1) and complement components that interact with resident non-cardiomyocyte cell types to activate the release of pro-inflammatory cytokines and chemokines [21-23]. Resident macrophages recognize HMGB1 through the receptor for advanced glycation end products and toll-like receptor 4 to release pro-inflammatory cytokines such as interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF- $\alpha$ ) [24,25]. These signals initiate leukocyte infiltration into the infarct region. Leukocytes clear necrotic tissue by releasing extracellular proteases, predominately serine proteases and matrix metalloproteinases (MMPs) that degrade ECM to remove cardiomyocyte debris [26-29]. Fibroblasts respond to and contribute to proinflammatory signaling during the inflammatory phase of MI. Fibroblasts isolated from the infarct region produce pro-inflammatory cytokines in response to MI relevant signaling stimuli such as TNF- $\alpha$  [30]. Stimulation of cultured cardiac myofibroblasts with IL-1 $\alpha$  induces secretion of IL-1 $\beta$ , TNF- $\alpha$ , and IL-6, indicating a positive feedback loop is initiated by cytokine stimulation [31,32].

In vivo, day 1 MI fibroblasts display a pro-inflammatory phenotype to contribute inflammatory cytokines and MMPs. Ex vivo transcriptomic analysis of

day 1 MI fibroblasts (cultured passage 2 cells) show expression of genes contributing to leukocyte infiltration, including *Il23a*, *Ccl5*, *Csf1*, and *Cxcl11*, and downregulation of anti-inflammatory *Il10* [10]. *Cx3c11*, *Ccl5*, and *Csf1* are highly upregulated in day 1 MI fibroblasts and return to day 0 concentrations by day 7 [10]. By evaluation of 17 signaling genes, *Tnfrsf9* signifies day 1 MI signaling. Pathway analysis of upregulated genes indicate positive regulation of macrophage chemotaxis and regulation of T helper 17 cells by Gene Ontology (GO) biological processes. Downregulation of anti-apoptotic Bcl2 indicate D1 MI fibroblasts have a pro-survival phenotype; this is coupled by an absence of proliferation, such that fibroblast numbers maintain but are not expanded. At MI day 1, cholesterol biosynthesis is the most downregulated GO biological process, indicative of a reduced need for cell membrane synthesis and consistent with the reduction in proliferation [10]. The shift to anti-migratory phenotype is coupled with decreased expression of a number of genes associated with migration, including *Cthrc1*, *Fgf2*, and *Fzd2* [10]. Thus fibroblasts, play a role in the initiation of, and the response to, inflammatory signaling. In response to the damage-induced inflammation, fibroblasts adopt an anti-migratory and anti-proliferative signature.

**Day 3 MI fibroblasts are anti-inflammatory and pro-angiogenic.** By MI day 3, fibroblasts in the infarct region enter the proliferative phase of cardiac remodeling. Fibroblast proliferation transcripts become upregulated, along with polarization to an anti-inflammatory and pro-angiogenic phenotype. Polarization to an anti-inflammatory phenotype suggests that fibroblasts play a role in transitioning from the inflammatory phase. Coupled with the increase in proliferation, cholesterol biosynthesis becomes the most upregulated GO biological process [10]. The upregulation in proliferation was due to increased expression of fibroblast proliferation marker cytoskeleton-associated protein 4 (*Ckap4*) at day 3, as *Ckap4* was increased while 9 other proliferation genes were not (*Ccnb2*, *Cenpa*, *Mcm2*, *Mik67*, *Pcna*, *Top2a*, *Tubb5*, *Tuba1a*, and *Tuba1b*) [10,33]. The van Rooij lab used single cell sequencing of freshly isolated cells from day 3 ischemia/reperfused myocardium and also identified *Ckap4* as a novel marker for activated fibroblasts [33]. Thus, despite the model (permanent occlusion vs. ischemia/reperfusion), isolation conditions (cultured to passage 1 on plastic vs. freshly used), or approach (RNA-sequencing vs. single cell sequencing), the results were reproducible across labs. Caspase 3 is also downregulated in MI day 3 cardiac fibroblasts, and this inhibition of apoptosis further contributes to an accumulation in fibroblast numbers. Day 3 MI fibroblasts use *Il4ra*, *Mapk7*, and *Nfkb1* signaling, reflective of the start in shift to anti-inflammation [10].

Expression of *Acta2* is linear in MI cardiac fibroblasts, with MI day 3 fibroblast showing about 50% of the MI day 7 expression [10]. In both *ex vivo* models and clinical studies, galectin-3 has been identified as a possible activator of the transforming growth factor  $\beta$ 1 (TGF $\beta$ 1)/*Acta2*/Collagen I pathway in cardiac fibroblasts, leading to ECM accumulation [34].

TGF $\beta$ 1 is a known pro-fibrotic growth factor and a key paracrine signal to induce fibroblast activation [35–37]. TGF $\beta$ 1 enhances ECM protein synthesis and is well-documented for its role in activating fibroblasts and cardiac repair following MI [38–42]. Activated fibroblasts further signal ECM accumulation through multiple signaling pathways, using platelet derived growth factor receptor-alpha and beta (PDGFR $\alpha$  and  $\beta$ ) in particular [43,44]. TGF $\beta$ 1 and vascular endothelial growth factor (VEGF), released by both anti-inflammatory macrophages and cardiac fibroblasts, increase connective tissue growth factor (CTGF) secretion by fibroblasts [4,45–49]. CTGF is also downregulated by IL-1 to yield lower activity in the inflammatory phase [50]. CTGF mAb treatment in MI mice at day 3 improved ejection fraction and survival at day 7. Long term treatment with CTGF blocking antibody limited fibrosis and cardiac hypertrophy [51]. The increase in VEGF in MI D3 fibroblasts also signals a stimulus from fibroblasts to initiate angiogenesis [52]. VEGF interacts with fibroblasts to promote angiogenesis in endothelial cells through both autocrine and paracrine mechanisms. VEGF can be induced by fibroblasts through fibroblast growth factor-2 (FGF-2) to stimulate endothelial tube formation [52–55].

Day 3 MI fibroblasts isolated from the infarct region of mice deficient in secreted protein acidic and rich in cysteine (SPARC) showed reduced expression of 22 ECM genes out of 84 measured [14]. Fibronectin, connective tissue growth factor, MMP-3, and tissue inhibitor of metalloproteinase-2 (TIMP-2) were all lower in expression (measured in passage 2–4 cells) compared to wild type day 3 MI fibroblasts. Fibroblast gene expression mirrored LV tissue protein for fibronectin, CTGF, and MMP-3 but not TIMP-2 [14]. Day 3 fibroblasts are proliferative, pro-angiogenic, show an increase in activation, and upregulate pro-fibrotic pathways that will continue to be upregulated in the maturation phase.

**Day 7 MI fibroblasts are ECM-producing and anti-angiogenic.** The maturation phase of cardiac remodeling occurs at day 7 and is characterized by ECM accumulation and scar formation. Fibroblasts are the major contributors of ECM during the maturation phase. Day 7 MI fibroblasts show reduced migration rates and a shift in adhesion preference from laminin to collagen IV [5]. Collagen synthesis increases 169% in fibroblasts isolated from the MI region, reflecting a shift to producing ECM to support the generation of the infarct scar. Collagen I and III

are predominant ECM proteins in the infarct scar [1]. There is a gradual increase in collagen in the infarct region for the first 6 days of MI followed by its subsequent gradual condensation into a scar with a steady increase in complexity [56]. Fibronectin (*Fn1*) is also upregulated in MI fibroblast gene expression [10]. Collagen complexity over the infarct scar further increases from day 7 to day 21 producing a robust contractile scar [15,56].

The expression of *Acta2* is linear over the first week, peaking at MI day 7 and returning towards day 0 values by MI day 28 [15,57]. *Acta2* expression indicates that fibroblasts have acquired a contractile phenotype. This phenotype is necessary to maintain structural integrity during the process of scar formation. Periostin is another marker of contractile fibroblasts that increases in the infarct region in the first week of MI [10,58]. Treatment with periostin locally improves cardiac physiology after MI but causes increased cardiac fibrosis suggesting periostin increases ECM production after MI [59]. At MI day 7, *Acta2* increases in cardiac fibroblasts isolated from the infarct zone compared to fibroblasts isolated from the remote zone, and remote zone fibroblasts show more *Acta2* expression than fibroblasts isolated from day 0 no MI hearts [5]. Of note, markers of origin shift with MI time. *Wt1* and *Twist1* are increased at MI day 7 compared to day 0 no MI and day 1 or 3 MI. *Tbx18* is increased at both MI days 3 and 7 compared to MI day 1, while *Tcf21* shows uniform expression across the time continuum [10]. Day 7 MI fibroblasts use *Pik3r3* and *Fgfr2* signaling. *Pdgfra* and *Cthrc1* denote the day 7 MI fibroblast, showing highest expression at this time. *Cthrc1* has been associated with tumor invasion and metastasis, working through upregulation of MMPs –7 and –9 [60]. While *Cthrc1* reverses collagen synthesis in keloid fibroblasts, its role in cardiac fibroblasts has yet to be divulged [61].

Fibroblasts convert from a pro-angiogenic state at MI day 3 to an anti-angiogenic profile at MI day 7 [10]. Fibroblast-mediated inhibition of angiogenesis is modulated by thrombospondin-1 (*Thbs1*). Treatment of cultured microvascular endothelial cells with day 7 MI fibroblast secretome reduces endothelial cell tube formation [10]. *Thbs1* is the highest upregulated fibroblast gene showing the most linear increase over MI time, and secreted *Thbs1* increases dramatically at day 7. Treatment with a *Thbs1* Ab significantly attenuates the decrease in endothelial cell tube formation. The increase in *Thbs1* could be related to the decrease in fibroblast-derived VEGF at day 7, as VEGF is a known negative regulator of *Thbs1* [10,62]. Like *Thbs1*, VEGF also negatively regulates *Thbs2* through hypoxia inducible factor-1 $\alpha$ , which would serve as an additional mechanism to prevent angiogenesis [63].



Secretomes collected from day 7 LV infarct fibroblast cultures show increased release of collagen I alpha 1 and 2 from cardiac fibroblasts, along with increases in the cross-linkers SPARC and lysyl oxidase (LOX) concentrations. Thus, the MI fibroblast secretome is a rich source of both ECM and its cross-linkers, and the fibroblast retains its secretome memory in culture. Secretome fractions collected under serum free conditions are also useful stimuli of other MI-relevant cell types, including inflammatory and endothelial cells, to examine cell cross-talk.

A number of modifications alter fibroblast responses to MI. MMP-28 deletion decreases collagen deposition and yields fewer myofibroblasts in the day 7 MI LV [13]. Collagen cross-linking is impaired in the absence of MMP-28 as a result of decreased expression and activation of lysyl oxidase. *Porphyromonas gingivalis* lipopolysaccharide pre-treatment exacerbates MI induction of Ccl12 in mice, which inhibits fibroblast activation, measured in vivo and ex vivo, to reduce scar formation and increase rupture rates [11]. Likewise, infusion of anti-inflammatory IL-10 starting at one day after MI reduces LV dilation and improves ejection fraction without affecting infarct area or mortality [12]. The change in cardiac physiology resulted from increased M2 anti-inflammatory gene expression in macrophages. Ex vivo, fibroblasts from the in vivo IL-10 treated group showed more fibroblast activation (increased proliferation, migration, and collagen production) that was the result of both direct and indirect effects on macrophage polarization. Importantly, the fibroblasts examined ex vivo in these studies were isolated, cultured in media with 10% fetal bovine serum, and used through passage 4. The major role of fibroblasts on day 7 is to deposit ECM to build a scar. Fibroblasts regulate angiogenesis by switching a pro-angiogenic phenotype at MI day 3 to an anti-angiogenic phenotype at MI day 7. Fibroblast activity, therefore, has direct effects on cardiac physiology through regulation of ECM and has indirect effects through regulation of other cardiac cell types.

**Long-term cardiac fibroblast activation to maintain the MI reset in homeostasis.** Beginning at MI day 7, fibroblasts begin to de-activate and return to their homeostatic roles sensing the ECM needs of the myocardium. Because there is a large replacement of myocytes with infarct scar, the ECM needs are greater than in the pre-MI state. Differences in cell physiology between cardiac fibroblasts before MI and long-term fibroblasts after MI is reflected primarily by the reset in cardiac homeostasis that occurs, including increased ECM production needed to maintain the infarct scar structure. The maintenance of the infarct requires constant sentinel feedback to the fibroblast. As late as 18 years after MI in humans, fibroblasts have been observed to be

present and functional in the infarct region [64]. The Blankenstein lab has put forth the concept that well-healed infarcts require the continued presence of a minimum number of fibroblasts to prevent adverse remodeling that progresses to heart failure [6,64]. Thus, strategies to eliminate fibroblasts from healed myocardium will likely prove detrimental.

## Conclusions

Fibroblasts are major players in scar formation following MI. Fibroblasts phenotypes shift through the different phases of remodeling. In the control setting, fibroblasts interact with the extracellular environment to maintain cardiac homeostasis. Following MI, fibroblasts immediately transition to an inflammatory phenotype, corresponding to the inflammatory phase of MI. During the proliferative phase, fibroblasts are pro-angiogenic. During the reparative phase, fibroblasts are responsible for laying down new ECM and downregulation of angiogenesis. Establishing the role of fibroblasts through different phases of cardiac remodeling has implications in targeting fibroblasts to improve scar formation.

There remains several gaps in our understanding of cardiac fibroblast roles after MI. Future studies are needed to understand what mechanisms induce fibroblast to polarize and whether polarization is bidirectional and reversible. Understanding the regulators of each fibroblast cell state will be important for both mechanistic insight and to develop targets that can adjust the system in controlled ways [65]. Computational studies will be informative in allowing a broad number of scenarios to be rapidly evaluated [66,67]. Cell to cell interactions play important roles in polarizing macrophages, and similar mechanisms likely work for fibroblasts [68]. Using spatial-omics approaches will help to define cell heterogeneity and its effect on ECM regulation by the fibroblast will provide a greater depth of knowledge [69]. While the cardiac phenotypes across the MI time continuum are now better defined, elucidating signaling patterns and cell interactions will allow us to modulate these cells, to assess if long-term MI outcomes can be predicted based on early fibroblast phenotypes. For example, in vitro renin-angiotensin-aldosterone signaling effects on cardiac fibroblasts are well understood, and a better understanding of its relationship with other signaling pathways such as Tgfb signaling is needed to predict how modulation will alter overall ECM accumulation in vivo [70]. In addition, more information is needed on the role of post-translational modifications. For example, fibronectin is citrullinated at cell-binding domain sites to alter fibroblast migration rates and support wound healing [71]. Thus, ECM records exposure to inflammation through post-translational modifications that are then read by fibroblasts to alter their cell physiology.

How these mechanisms may work in cardiac fibroblasts has not been examined. Limiting the inflammatory phenotype and promoting the reparative phenotype could potentially improve the process of scar formation. In contrast, enhancing the reparative phenotype could cause excess collagen deposition and fibrosis, leading to reduced cardiac function. Thus, the right balance in ECM deposition is needed for the happy medium of providing enough structure. Acquiring new knowledge of fibroblast phenotypes in relation to MI time will allow us to more accurately target fibroblasts to improve long-term outcomes.

### CrediT authorship contribution statement

**Michael J. Daseke II:** Visualization, Writing - original draft, Writing - review & editing. **Mavis A.A. Tenkora:** Visualization, Writing - original draft, Writing - review & editing. **Uendra Chalise:** Visualization, Writing - original draft, Writing - review & editing. **Shelby R. Konfrst:** Visualization, Writing - original draft, Writing - review & editing. **Merry L. Lindsey:** Visualization, Writing - original draft, Writing - review & editing

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