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Cardiosphere-Derived Cells Reverse Heart Failure With Preserved Ejection Fraction in Rats by Decreasing Fibrosis and Inflammation

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VISUAL ABSTRACT

HIGHLIGHTS

- The pathogenesis of heart failure with a preserved ejection fraction (HFpEF) is unclear.
- Cardiosphere-derived cells (CDCs) are heart-derived cell products with anti-fibrotic and anti-inflammatory properties, which have been implicated in HFpEF.
- Dahl salt-sensitive rats were fed a high-salt diet for 6 to 7 weeks and randomized to receive intracoronary CDCs or placebo.
- Following CDC treatment, diastolic dysfunction resolved in treated rats but not in the placebo group. Treatment with CDCs also lowered LV end-diastolic pressure, decrease lung congestion, and enhance survival.
- CDC treatment decreased LV fibrosis and inflammatory infiltrates, and reversed many of the transcriptomic changes associated with HFpEF, but had no effect on cardiac hypertrophy.
- By selectively reversing inflammation and fibrosis, CDCs may be beneficial in the treatment of HFpEF.

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Hypertensive LV hypertrophy with HFpEF (15) is a consequence of various cardiovascular risk factors, especially hypertension, extracardiac comorbidities, and aging. The net result is impaired diastolic relaxation and filling of the left ventricle, increased myocardial stiffness, impaired vascular compliance, and increased diastolic pressure. Myocardial fibrosis and inflammation have been associated with HFpEF (9-14) and with the transition from hypertensive left ventricular (LV) hypertrophy without HFpEF to hypertensive LV hypertrophy with HFpEF (15). Cardiosphere-derived cells (CDCs) are heart cell products with anti-fibrotic, anti-inflammatory, and angiogenic properties (16-20). CDCs, which are currently in phase 2 human trials for scar reduction after myocardial infarction (5), have been shown to be beneficial in models of ischemic (17,18,21) and non-ischemic cardiomyopathy (16). Thus, we wondered whether CDCs might have disease-modifying activity in HFpEF.

Dahl salt-sensitive (DS) rats develop hypertension, hypertrophy, and, eventually, HFpEF on a high-salt diet (22-26). Increased fibrosis and inflammation underlie the development of HFpEF, with resultant cachexia, pulmonary congestion, and accelerated mortality (22,26,27). Therefore, this model has been widely used to test new treatments for HFpEF (23,27-31). Here, we tested the efficacy of CDCs in improving LV structure and function and overall outcome in DS rats with HFpEF.

**METHODS**

An expanded “Methods” section is available in the Supplemental Appendix.

DS rats (Charles River, Wilmington, Massachusetts) were fed a 0.3% NaCl (low-salt) diet until 7 weeks of age. At that time, the diet was switched to an 8% NaCl (high-salt) diet in 54 rats by random assignment. DS rats fed the low-salt diet constituted the control group (n = 18). At 13 to 14 weeks of age, rats on the high-salt diet were randomized to receive intracoronary CDCs or placebo.
receive allogeneic rat CDCs ($5 \times 10^5$ resuspended in 100 μl phosphate-buffered saline) (Figure 1A) or vehicle (phosphate-buffered saline). CDCs were grown from a freshly explanted Wistar-Kyoto rat heart as previously described (19) (Figure 1B).

Echocardiography was performed at baseline, before treatment, and 1 and 4 weeks after treatment to assess systolic and diastolic function. Invasive hemodynamic measurements were performed at endpoint to record systemic pressure and LV pressures and volumes. Pressure-volume loops were generated from these recordings. Rats were then euthanized and hearts were harvested for analysis. Additional follow-up (7 rats in each group, randomly chosen from the rats alive at 4 weeks) was performed for extended survival analysis (up to 6 weeks) to investigate the longer term effects of treatment.

**STATISTICAL ANALYSIS.** Continuous variables are presented as mean ± SD in the text and mean ± SE in the figures. Categorical variables are expressed as absolute numbers and percentages. Normal distribution of variables was assessed using the Kolmogorov-Smirnov test. If normality was established, independent groups (n = 2) were compared using unpaired Student t test, and multiple groups were compared using 1-way analysis of variance. For variables not normally distributed, the Mann-Whitney U test was used for comparisons of 2 groups, and the Kruskal-Wallis test.
was used to compare multiple groups. Bonferroni correction was applied to every pairwise comparison performed after analysis of variance or the Kruskal-Wallis test. Survival analysis was performed using Kaplan-Meier analysis. A p value <0.05 was considered to indicate statistical significance.

RESULTS

BLOOD PRESSURE AND CARDIAC HYPERTROPHY. Table 1 shows characteristics of the high-salt and control animals at baseline and after 6 weeks of diet (13 weeks of age). As expected (25), rats fed a high-salt diet developed hypertension and cardiac hypertrophy after 6 weeks, but low-salt control rats did not. Those changes were associated with diastolic dysfunction, as shown by a decreased ratio of early (E) to late (A) ventricular filling velocity (E/A ratio) by echocardiography (1.7 ± 0.2 vs. 1.2 ± 0.2, p < 0.001), without any changes in LV volumes, LV ejection fraction (LVEF) or fractional area change (Table 1).

ECHOCARDIOGRAPHIC STUDIES: CDCs NORMALIZE E/A RATIO. Having confirmed the presence of cardiac hypertrophy and diastolic dysfunction, we randomly allocated rats to intracoronary CDC or vehicle infusion. Figure 1C shows representative images of transmitial flow at endpoint in control, placebo-treated, and CDC-treated animals. Pooled data (Figure 1D) revealed that after 6 weeks of diet but before treatment, E/A ratios were similar in the placebo and CDC groups but lower than in control rats. Likewise, left atrial size was higher in the high-salt-fed rats compared with control rats, indicating already increased LV filling pressure. After intracoronary infusion of CDCs (but not placebo), E/A ratio increased over time (Figure 1D), a change that was evident as soon as 1 week after treatment. At endpoint, E/A ratios had returned to control levels in CDC-treated animals (1.7 ± 0.2 for CDC-treated vs. 1.8 ± 0.16 for control rats, p = 0.36), whereas they remained depressed in placebo-treated animals (1.2 ± 0.3, p < 0.001 vs. CDC-treated rats and vs. control rats), indicating a likely normalization of LV relaxation with CDC treatment (an interpretation verified later by hemodynamic recordings). In addition, left atrial dimensions kept increasing in the placebo animals, while CDC treatment halted left atrial enlargement. In contrast, LVEF (measured in long-axis views) (Figures 1E and 1F), fractional area change (from short-axis views) (Figures 1G and 1H), and LV volumes (Figures 1I and 1J) were equivalent in all groups.

HEMODYNAMIC STUDIES: CDC TREATMENT NORMALIZES LV RELAXATION AND PREVENTS ELEVATION OF LV END-DIASTOLIC PRESSURE. Figure 2A shows representative recordings of pressure-volume loop families at endpoint. The time constant of isovolumic LV pressure fall (tau) was prolonged in placebo-treated animals compared with CDC-treated animals (21 ± 8 s vs. 13 ± 1 s in control rats [p = 0.011] and 14 ± 4 s in CDC-treated rats [p = 0.006]) (Figure 2B) and control rats, while –dP/dt minimum was lower, indicating impaired relaxation (Figure 2C) without changes in dP/dt maximum (Figure 2D). In parallel, pressure-volume loop analyses confirmed that LV distensibility was decreased in the placebo-treated animals, as demonstrated by the steeper slope of the end-diastolic pressure-volume relationship in placebo-treated animals compared with CDC-treated and control animals (Figure 2E), again without changes in the end-systolic pressure-volume relationship (Figure 2F). LVEDP was 2-fold higher in placebo-treated than in CDC-treated and control animals (17 ± 10 mm Hg vs. 9 ± 4 mm Hg in control rats [p = 0.015] and 8 ± 3 mm Hg in CDC-treated rats [p = 0.002]) (Figure 2G). The normalization of LVEDP and tau in CDC-treated rats confirms that the increase of E/A ratio over time in this group was due to normalization of LV relaxation rather than to progression toward a pseudonormal pattern of transmitial flow (which

| TABLE 1 Characteristics of Rats Fed High- and Low-Salt Diets Before (Baseline) and After 6 Weeks of Diet |
|-----------------------------------|------------------|------------------|------------------|-------------------|------------------|------------------|------------------|------------------|
|                                  | Baseline         |                   | 6 Weeks          |                   | 6 Weeks          |                   | 6 Weeks          |                   |
|                                  | Low Salt         | High Salt        | p Value          | Low Salt          | High Salt        | p Value          | Low Salt          | High Salt        |
| SBP (mm Hg)                      | NA               | NA               |                  | 133 ± 23          | 188 ± 17*        | 0.001            | 92 ± 15           | 150 ± 12*        | 0.001            |
| DBP (mm Hg)                      | NA               | NA               |                  | 71.6 ± 3.8        | 74.4 ± 5.9       | 0.10             | 62.3 ± 4.6        | 63.2 ± 5.9       | 0.61             |
| LVEF (%)                         | 73.1 ± 4.8       | 73.0 ± 5.1       | 0.90             | 71.6 ± 3.8        | 74.4 ± 5.9       | 0.10             | 62.3 ± 4.6        | 63.2 ± 5.9       | 0.61             |
| FAC (short axis) (%)             | 64.1 ± 3.0       | 64.2 ± 4.9       | 0.95             | 62.3 ± 4.6        | 63.2 ± 5.9       | 0.61             | 62.3 ± 4.6        | 63.2 ± 5.9       | 0.61             |
| AW thickness (mm)                | 1.2 ± 0.1        | 1.2 ± 0.1        | 0.54             | 1.2 ± 0.1         | 1.8 ± 0.8*       | <0.001           | 1.3 ± 0.1         | 2.0 ± 0.6*       | <0.001           |
| PW thickness (mm)                | 1.3 ± 0.1        | 1.3 ± 0.1        | 0.27             | 1.3 ± 0.1         | 2.0 ± 0.6*       | <0.001           | 1.3 ± 0.1         | 2.0 ± 0.6*       | <0.001           |
| LVEDV (ml)                       | 330 ± 46         | 329 ± 70         | 0.93             | 484 ± 112         | 510 ± 110        | 0.42             | 484 ± 112         | 510 ± 110        | 0.42             |
| LVESV (ml)                       | 89 ± 22          | 89 ± 28          | 0.98             | 142 ± 47          | 130 ± 50         | 0.37             | 142 ± 47          | 130 ± 50         | 0.37             |
| E/A ratio                        | 1.7 ± 0.2        | 1.7 ± 0.3        | 0.66             | 1.7 ± 0.2         | 1.2 ± 0.2*       | <0.001           | 1.7 ± 0.2         | 1.2 ± 0.2*       | <0.001           |
| Left atrial area (mm²)           | 13.7 ± 1.9       | 14.1 ± 1.9       | 0.55             | 17.9 ± 1.7        | 21.2 ± 2.9*      | <0.001           | 17.9 ± 1.7        | 21.2 ± 2.9*      | <0.001           |
| Heart weight (g)                 | NA               | NA               |                  | 1.42 ± 0.14       | 1.67 ± 0.10*     | 0.03             | 1.42 ± 0.14       | 1.67 ± 0.10*     | 0.03             |
| Heart weight/body weight (mg/g)  | NA               | NA               |                  | 4.1 ± 0.4         | 5.2 ± 0.5*       | 0.016            | 4.1 ± 0.4         | 5.2 ± 0.5*       | 0.016            |

Values are mean ± SD. *p < 0.05 between high-salt and low-salt groups at 6 weeks. AW = anterior wall; DBP = diastolic blood pressure; E/A ratio = ratio of early to late ventricular filling velocity; FAC = fractional area change; LVEDV = left ventricular end-diastolic volume; LVEF = left ventricular ejection fraction; LVESV = left ventricular end-systolic volume; NA = not available; PW = posterior wall; SBP = systolic blood pressure.
would have been associated with increased LVEDP and tau \cite{32}.

We did not observe any differences in blood pressure or heart rate between CDC- and placebo-treated animals that could have confounded relaxation and LVEDP measurements (although blood pressure was lower in the control group, as expected) (Figures 2H to 2J). Thus, the improvements in diastolic function were not due to antihypertensive or chronotropic effects of CDCs.

**CDC TREATMENT IMPROVES SURVIVAL AND DECREASES LUNG CONGESTION.** Consistent with the improvement of diastolic function, we observed a dramatic improvement of survival in CDC-treated rats (Kaplan-Meier survival curves) (Figure 3A) (log-rank
p = 0.027). Post-mortem lung weight and lung weight/body weight ratios were higher in placebo-treated rats, indicative of pulmonary congestion (Figure 3B). In parallel, Figure 3C shows that animals treated with CDCs resumed some physiological weight gain, while placebo rats lost weight, presumably because of cardiac cachexia (an impression that was confirmed visually).

**MECHANISM.** Improvement of LV relaxation is not associated with quantitative changes in cardiac hypertrophy. LV hypertrophy (both macroscopic and cellular) can occur with or without diastolic dysfunction. We quantified cardiac hypertrophy using LV wall thickness by echocardiography, heart weight, and cardiomyocyte cross-sectional area. Notably, the CDC-related improvement in diastolic function was not due to a decrease in cardiac hypertrophy: wall thickness by echocardiography (Figure 4A), as well as post-mortem heart weight and cardiomyocyte cross-sectional area (Figure 4B), remained equivalent in the CDC and placebo groups. Thus, CDCs were salutary without decreasing cardiac hypertrophy.

**Antifibrotic effect of CDCs.** Fibrosis is increased in HFpEF (10,12,33). We assessed fibrosis using picrosirius red staining for total collagen and semiquantitative reverse transcriptase polymerase chain reaction to measure transcript levels for collagen 1 and 3. Figure 5A shows representative images of hearts stained with picrosirius red. Overall LV and right ventricular fibrosis was 2-fold higher in placebo- versus CDC-treated rats; fibrosis in the latter approached control values (Figures 5B and 5C). Concomitantly, collagen 1 and collagen 3 in the left ventricle (quantified by western blot) were higher in placebo-treated rats than in control or CDC-treated rats (Figure 5D). Moreover, cardiac myofibroblasts increased dramatically in placebo-treated, but not in CDC-treated, DS rats (Figure 5E). Also, transcript levels of matrix metalloproteinase (MMP)-2, MMP-7, MMP-9, and tissue inhibitor of metalloproteinase (TIMP)-1 as well as collagen 1A1 and collagen 3 were higher in the placebo-treated animals compared with the control and CDC-treated animals (which had similar levels) (Supplemental Figure 1). These increased transcript levels are suggestive of increased extracellular matrix turnover associated with HFpEF, which is normalized by CDC treatment. Because we did not measure the extent of fibrosis before treatment, we cannot distinguish between CDC-induced prevention of new fibrosis and regression of established fibrosis in the present study. However, previous work in chronic myocardial infarction models (20,21,34) and in humans (35,36) has shown that CDCs (and cardiospheres) can reduce established scar.

**Attenuation of inflammation.** HFpEF is associated with increased levels of circulating cytokines and infiltration of macrophages and other inflammatory cells in the heart (9). Quantification of cytokines in the serum revealed lower levels of proinflammatory and profibrotic cytokines in CDC-treated rats compared with placebo-treated rats; the levels in CDC-treated rats were comparable with those in...
control rats (Figure 6A). Among those cytokines, some have been linked to the development of HFpEF (especially monocyte chemotactic protein-1, interleukin-6, and tumor necrosis factor-α) and to the accumulation of collagen (TIMP-1) (12,37). CDC treatment was also associated with a 2-fold reduction of macrophages (CD68-positive cells) and leukocytes (CD45-positive cells) in the heart compared with placebo, approaching control levels (Figure 6B).

**Vessel density and cell proliferation.** Because microvascular rarefaction has been associated with HFpEF in numerous studies (38–40), we investigated...
arteriolar and capillary density in the left ventricle (Figure 7A). Both vascular densities were lower in placebo-treated rats compared with control rats (Figures 7B and 7C); CDC treatment normalized arteriolar density and significantly increased capillary density compared with placebo, although capillary density did not reach the value measured in control rats. Parallel measurements of cell proliferation using
Ki67 immunostaining (Figure 7D) revealed that CDCs stimulated cardiomyocyte proliferation (cells positive for both α-sarcomeric actinin and Ki67) (Figure 7E). In contrast, the number of proliferating fibroblasts (Ki67-positive, vimentin-positive cells) was greatly increased in placebo-treated (but not CDC-treated) high-salt DS rat hearts relative to low-salt control rats (Figure 7F).

**Next-generation ribonucleic acid sequencing.**

Next-generation sequencing was performed in the
**FIGURE 7** Microvascular Density and Cardioproliferation Enhanced by CDCs, While Fibroblast Proliferation Is Suppressed

(A) Immunostaining for von Willebrand factor (VWF) and smooth muscle actin (SMA) in control, placebo-treated, and cardiosphere-derived cell (CDC)-treated rats. CDC treatment increases arteriolar (B) and capillary (C) density in the left ventricle. (D) Immunostaining for Ki67 and α-actinin (SA) and for Ki67 and vimentin in control, placebo-treated, and CDC-treated rats. CDC treatment increased cardiomyocyte (CM) proliferation (E) and decreased the proliferation of fibroblasts (F) compared with placebo. n = 5 in each group. †p < 0.05 versus control and CDC-treated rats, both by analysis of variance; ‡p < 0.05 versus control and placebo-treated rats. DAPI = 4',6-diamidino-2-phenylindole.
3 groups. Supplemental Figures 2A to 2C show head-to-head pairwise comparison of gene expression in the 3 groups. The heat maps reveal that the HFpEF phenotype is associated with major global changes in gene expression, as shown by the comparison between placebo and control groups (Supplemental Figure 2A). More important, the comparison between CDC- and placebo-treated rats (Supplemental Figure 2B) reveals that CDC treatment dramatically changed gene expression. Interestingly, >300 genes whose expression was up- or downregulated in HFpEF (i.e., in high-salt placebo hearts) had their expression levels “rescued” by CDC treatment (Figure 8A). Some of these transcript changes involved genes that underlie HFpEF-related pathophysiologic features that we and others have identified (a nonexhaustive list is shown in Figure 8B). Indeed, key genes involved in fibrosis, inflammation, and macrophage signaling, or associated with the consequences of HFpEF (brain natriuretic and atrial natriuretic peptides), were upregulated in placebo hearts but returned fully or partially to control levels after CDC treatment. These profound changes in the transcriptome reveal HFpEF-related activation, and CDC-induced inhibition, of key disease-associated signaling and effector pathways (Figure 8C).

**DISCUSSION**

The challenge of HFpEF is increasing as the population ages and comorbidities become more
prevalent. The HFpEF hospitalization rate is now greater than that for heart failure with reduced LVEF (3). So far, no treatment for HFpEF has proved effective (6). Here, we have demonstrated that cell therapy by CDCs can reverse the functional abnormalities of HFpEF and improve survival in a rat model of hypertension-induced HFpEF. The CDC-induced reversal of HFpEF occurred without a reduction in either blood pressure or cardiac hypertrophy. The selective correction of functional HFpEF abnormalities creates an unprecedented opportunity for mechanistic insights. Potentially causal pathways (i.e., those that accompany the abnormalities in HFpEF) can now be distinguished from those that are merely associative. Our findings support the concept that fibrosis and inflammation are causative in HFpEF (10): reductions in those 2 pathophysiological processes underlie the resolution of HFpEF, while hypertrophy and hypertension remain unchanged.

Cardiac hypertrophy has long been thought to be the linchpin in HFpEF (8,41,42). However, several recent studies in animal models and humans have implicated inflammation and collagen infiltration (10,12-14,43-45). Hypertension and other comorbidities can favor a systemic proinflammatory state with high circulating cytokine levels, including interleukin-6, tumor necrosis factor-α, and monocyte chemotactic protein-1 (9,10,37). Inflammation leads to activation, recruitment, and transendothelial migration of leukocytes and monocytes or macrophages into the heart. These inflammatory cells contribute to LV fibrosis by promoting the differentiation of fibroblasts into myofibroblasts (33,46,47). The resulting increase in LV collagen content is the main contributor to the increase in passive myocardial fiber stiffness, a major component of diastolic impairment in HFpEF (12). The observed phenotypic improvements after CDC treatment were associated with decreases in circulating inflammatory cytokines (including interleukin-6, tumor necrosis factor-α, and monocyte chemotactic protein-1) and less myocardial inflammation. In addition, myofibroblast infiltration, collagen content, and collagen production were increased in placebo-treated animals but fell markedly after CDC treatment. The parallel decrease in transcripts for MMPs and TIMPs (Supplemental Figure 1) suggests, but does not prove, increased extracellular matrix turnover in the placebo-treated animals that is normalized by CDC treatment. Increased extracellular matrix turnover in HFpEF has been described, and MMPs and TIMPs have been suggested as biomarkers for the diagnosis and prognosis of HFpEF (48-50). Taken together, these findings strengthen the hypothesis that proinflammatory and profibrotic stimuli play a major role in the development of HFpEF (10). Furthermore, our results suggest that modulating those stimuli may improve HFpEF phenotype and outcomes. The mechanism whereby CDCs modify inflammation and fibrosis clearly involves major changes in gene expression (Figure 8C). Such changes are long-lasting, as the transcriptome was analyzed 4 weeks after CDC injection (at which point injected allogeneic cells are no longer detectable) (18). Our working hypothesis posits that CDCs secrete exosomes laden with micro-ribonucleic acids and other noncoding ribonucleic acids that collectively mold the target transcriptome. Although in-depth exploration of this hypothesis is beyond the scope of this initial report, multiple lines of evidence in other models show that exosomes mediate the benefits of CDCs and modify phenotype and gene expression in recipient cells (51-53). We are intrigued by the possibility that the drastic changes in the expression of the genes involved in fibrosis and inflammation seen here may be in vivo manifestations of exosome-mediated phenotypic conversions such as those we have described in skin fibroblasts (52).

It is noteworthy that no changes in the magnitude of cardiac hypertrophy were observed after CDC treatment. Cardiac hypertrophy assessed using 3 different techniques (echocardiography, heart weight, and cardiomyocyte cross-sectional area) was present and virtually identical in both high-salt groups at endpoint but not in control rats. Here, decreased inflammation and fibrosis underlie the resolution of HFpEF, despite persistent hypertrophy and hypertension. Thus, attenuation of cardiac hypertrophy is not required to normalize diastolic function. Regarding the mechanical properties of the cardiomyocytes, we observed that CDCs enhance cardiomyocyte (but not fibroblast) proliferation. Nevertheless, the absolute number of new myocytes remains low, and cardiomyocytes remain hypertrophic after CDC treatment. Thus, the correction of altered mechanical properties is more likely related to intrinsic changes in preexisting cardiomyocytes than to a dominant effect of newly generated cardiomyocytes. Our ongoing characterization of cardiomyocytes isolated from the various groups is consistent with this prediction, but such studies are beyond the scope of this initial report.
Microvascular dysfunction and rarefaction have been reported as additional contributors to HFpEF. Several studies have shown that even in the absence of coronary artery disease, patients with HFpEF have fewer microvessels and lower coronary reserve (38–40). In addition to their anti-inflammatory and anti-fibrotic properties, CDCs are able to promote the growth of new vessels (18,21). We observed increases in the numbers of arterioles and capillaries in the left ventricle after CDC treatment (Figure 7). Although the vascular findings here are limited to histologic findings, we have previously demonstrated that cardiosphere-related increases in vessels by histology were associated with augmented myocardial perfusion and coronary reserve (54).

**STUDY LIMITATIONS.** HFpEF is a multifactorial disease involving aging and cardiovascular risk factors. Although DS rats reproduce most of the key features of HFpEF (including hypertension, inflammation, fibrosis, and microvascular rarefaction), some typical contributors to this disease (especially aging) are absent in this model. Also, our follow-up was relatively short. Because death and/or progression toward systolic dysfunction occurs in DS rats around 19 to 20 weeks of age (55,56), we decided to set the endpoint before the terminal decrease in LVEF, which might otherwise have confounded the analysis of HFpEF. Therefore, we have no information (other than the observed mortality benefit at 6 weeks) regarding the potential long-term persistence of the benefit. Regarding remodeling of the extracellular matrix, we have shown directionally appropriate changes in various transcripts and proteins, but we have not performed zymography to directly evaluate MMP activity. Finally, we have yet to demonstrate the involvement of exosomes and micro-ribonucleic acids in the dramatic changes observed after CDC treatment here. For now, the proposed mechanisms remain at the level of plausibility, as bolstered by previous studies (51,52).

**CONCLUSIONS**

CDCs normalized LV relaxation and improved survival in a rat model of HFpEF, without blunting hypertension or hypertrophy. Given that CDCs are already in advanced clinical testing for other indications (57), the present findings motivate clinical trials of CDCs in HFpEF.

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**PERSPECTIVES**

**COMPETENCY IN MEDICAL KNOWLEDGE:**

Intracoronary CDC administration normalizes diastolic function and improves survival in rats with HFpEF. Reversal of inflammation and fibrosis, but not attenuation of cardiac hypertrophy, underlies these functional benefits.

**TRANSLATIONAL OUTLOOK:** Given that CDCs are already in advanced clinical testing for other indications, the present study motivates clinical trials of CDCs in HFpEF. Also, the selective correction of functional HFpEF abnormalities observed here creates an unprecedented opportunity for mechanistic insights.

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**KEY WORDS** animal models, cell therapy, diastolic function, heart failure with preserved ejection fraction

**APPENDIX** For supplemental figures, please see the supplemental appendix.
Extracellular Matrix Hydrogel Promotes Tissue Remodeling, Arteriogenesis, and Perfusion in a Rat Hindlimb Ischemia Model

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VISUAL ABSTRACT

HIGHLIGHTS
- Although surgical and endovascular revascularization can be performed in patients with peripheral arterial disease (PAD), 40% of patients with critical limb ischemia do not have a revascularization option.
- The efficacy of an injectable tissue-specific skeletal muscle extracellular matrix (ECM) hydrogel and a human umbilical cord-derived ECM hydrogel were examined in a rodent hindlimb ischemia model.
- Although both biomaterials increased tissue perfusion 35 days post-injection, likely through arteriogenesis, the skeletal muscle ECM hydrogel more closely matched healthy tissue morphology.
- Transcriptomic analysis indicates the skeletal muscle ECM hydrogel shifted the inflammatory response, decreased necrosis/apoptosis, and increased blood vessel and muscle development.

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Although surgical and endovascular revascularization can be performed in peripheral arterial disease (PAD), 40% of patients with critical limb ischemia do not have a revascularization option. This study examines the efficacy and mechanisms of action of acellular extracellular matrix-based hydrogels as a potential novel therapy for treating PAD. We tested the efficacy of using a tissue-specific injectable hydrogel derived from decellularized porcine skeletal muscle (SKM) and compared this to a new human umbilical cord-derived matrix (hUC) hydrogel, which could have greater potential for tissue regeneration because of the younger age of the tissue source. In a rodent hindlimb ischemia model, both hydrogels were injected 1-week post-surgery and perfusion was regularly monitored with laser speckle contrast analysis to 35 days post-injection. There were significant improvements in hindlimb tissue perfusion and perfusion kinetics with both biomaterials. Histologic analysis indicated that the injected hydrogels were biocompatible, and resulted in arteriogenesis, rather than angiogenesis, as well as improved recruitment of skeletal muscle progenitors. Skeletal muscle fiber morphology analysis indicated that the muscle treated with the tissue-specific SKM hydrogel more closely matched healthy tissue morphology. Whole transcriptome analysis indicated that the SKM hydrogel caused a shift in the inflammatory response, decreased cell death, and increased blood vessel and muscle development. These results show the efficacy of an injectable ECM hydrogel alone as a potential therapy for treating patients with PAD. Our results indicate that the SKM hydrogel improved functional outcomes through stimulation of arteriogenesis and muscle progenitor cell recruitment. (J Am Coll Cardiol Basic Trans Sci 2016;1:32–44) © 2016 The Authors. Published by Elsevier on behalf of the American College of Cardiology Foundation. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).
investigated the tissue-specific SKM hydrogel compared to a new non-tissue-specific hydrogel derived from decellularized human umbilical cord matrix (hUC) in a rodent hindlimb ischemia model with chronically reduced perfusion to test their ability to improve hindlimb tissue perfusion, neovascularization, and muscle fiber remodeling.

MATERIALS AND METHODS

Full Methods are included in the Supplemental Appendix. These include: ECM hydrogel development and characterization, in vitro proliferation and migration, hindlimb ischemia surgery and biomaterial injection, hindlimb functional perfusion measurements, histological analysis, microarray analysis and quantitative polymerase chain reaction, and statistical analysis. All experiments in this study were performed in accordance with the guidelines established by the Institutional Animal Care and Use Committee at the University of California, San Diego, and the American Association for Accreditation of Laboratory Animal Care. Briefly, unilateral hindlimb ischemia was induced in female Sprague Dawley rats by removing a 2 cm segment of the femoral artery and vein. A biomaterial alone, SKM (n = 9) or hUC (n = 10), or saline control (n = 11) was injected into the gracilis muscle distal from the vessel ligation 7 days post-surgery. Ischemia was confirmed and monitored with laser speckle contrast analysis (LASCA) over 35 days post-injection. SKM alone was compared to saline at 3 and 10 days post-injection with histology and whole transcriptome analysis. Data are mean ± SEM unless otherwise noted. Significance was accepted at p < 0.05.

RESULTS

DECELLULARIZED TISSUES CAN BE PROCESSED INTO INJECTABLE HYDROGELS AND DIFFERENTIALLY AFFECT CELL MIGRATION AND PROLIFERATION IN VITRO. For comparison to the porcine SKM hydrogel, we developed a new non-tissue-specific injectable biomaterial from decellularized human umbilical cord tissue (hUC). Biomaterial characterization, including quantitative mass spectrometry analysis (Supplemental Figure 1, Supplemental Tables 1 to 4), and in vitro
FIGURE 2 Hindlimb Tissue Perfusion and Perfusion Kinetics

(A) Hindlimb perfusion measurements over the 42-day period for animals treated with either the skeletal muscle matrix (SKM) ($n = 9$), human umbilical cord matrix (hUC) ($n = 10$) hydrogels, or saline ($n = 11$). Readings are shown after the animal had been under anesthesia for 5 min, 10 min, and after reaching perfusion equilibrium. Vertical dotted line indicates time of treatment injection on day 0. (B) Individual animal perfusion readings on day 35. (C) Example representative perfusion images for each treatment group after the animal was under anesthesia for 10 min. Healthy limbs are on the left and ischemic/treated limbs are on the right. Note: Because the units for perfusion are arbitrary, color comparisons cannot be performed between 2 different animals. (D) Perfusion kinetics for pre-surgery and post-surgery. (E) Pre-injection perfusion kinetics on day –2 (pre-injection). (F) Final perfusion kinetics on day 35. A 2-way analysis of variance was conducted to compare within and between treatment groups. $p < 0.05$ for SKM compared to saline using Tukey post hoc test.
effects on cell proliferation and migration (Supplemental Figure 2) are described in the Supplemental Appendix.

**INJECTABLE ECM HYDROGELS INCREASE HINDLIMB TISSUE PERFUSION.** To mimic chronically reduced perfusion associated with PAD, a rat hindlimb ischemia model was utilized (Figure 1A). ECM hydrogels or saline were injected 1 week after ischemia induction (day 0), and tissue perfusion was assessed utilizing LASCA (Figure 1B) (13). LASCA allowed for continuous monitoring and instantaneous full-field analysis of an animal with readings lasting at least 20 min to reach perfusion equilibrium (Figure 1C). Percent perfusion of the ischemic limb to healthy limb was calculated once the animal had been under anesthesia for 5 min, 10 min, and then after reaching perfusion equilibrium, to allow for comparison to currently published laser Doppler data, which is only analyzed at a single time point post-anesthesia induction, typically around 10 min (14,15). Both the pre- and immediately post-surgery perfusion measurements were compared between treatments group and shown to not be statistically different by a 2-way analysis of variance (Figure 1D).

Both of the injected hydrogels alone, SKM or hUC, led to a significant increase in percent perfusion compared to saline as early as day 21 post-injection (Figure 2A) and this improvement was maintained until the end of the study on day 35 (Figures 2B and 2C). For example, the day 35 measurements after being under anesthesia for 10 min (a typical time for laser Doppler measurements) (14) showed the ischemic damaged limb to be perfused at 65.7 ± 13.3% for saline, 96.4 ± 13.8% for SKM (p < 0.001, compared to saline), and 84.2 ± 14.3% for hUC (p < 0.05, compared to saline). All 3 perfusion assessments, at 5 and 10 min under anesthesia and at equilibrium, showed that saline animals plateaued 7 days post-injection; subsequent measurements at days 14, 21, 28, and 35 were not statistically different than at day 7 (Figure 2A) (p > 0.05). Animals injected with hUC hydrogel also did not significantly improve from their perfusion at day 7 (Figure 2A) (p > 0.05). In contrast, perfusion in SKM animals was significantly higher at day 35 compared to day 7 at the 10-min reading, and at days 21, 28, and 35 compared to day 7 at equilibrium (Figure 2A) (p < 0.05).

Due to the use of LASCA we were able to monitor dynamic perfusion while the animals were under anesthesia. The animals were initially anesthetized with 5% isoflurane and then transferred to 2.5% when the perfusion reading started. Decreasing the level of isoflurane creates an increase in cardiac output and blood perfusion in the peripheral skeletal muscle (16). Thus, the perfusion increased in the hindlimbs as the animal rested on the deck, as illustrated in Figure 1C. From this data, a new

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**FIGURE 3 Histological Assessment of Arteries and Capillaries**

(A) Capillary density quantification. (B) There were no differences in arteriole density between all groups, but (C) the average arteriole diameter (p = 0.0389) and (D) density of arterioles with diameter >75 μm (p = 0.0344) was significantly increased in the skeletal muscle matrix (SKM) group. Data from the healthy limb is shown as a reference. (E) Representative image of capillaries stained by alkaline phosphatase for the SKM treatment group. (F) Example arteriole staining with anti-αSMA (red) and nuclei (blue) for saline (n = 11), (G) SKM (n = 9), and (H) human umbilical cord matrix (hUC) (n = 10). *p < 0.05 and scale bars are 100 μm.
parameter called perfusion kinetics ratio can be reported to show the rate of change of perfusion over time. This was calculated as a ratio of the slopes from the linear region of the perfusion measurements (indicated by dotted lines on Figure 1C) from the ischemic foot to the healthy foot. Perfusion kinetics ratios that deviate from 1 thus illustrate differences in perfusion between the 2 legs, with values <1 demonstrating a slower response to changes in cardiac output in the ischemic limb. The healthy animals pre-surgery had symmetric perfusion rates in each limb, or a perfusion kinetics ratio near 1 (Figure 2D). Immediately post-surgery, the ischemic damaged tissue showed a decreased rate of change in perfusion, which is indicated by the perfusion kinetics ratio being close to zero for all groups (Figure 2D). By day 5 post-ischemia (2 days pre-injection), the animals had partially recovered due to endogenous healing mechanisms and produced consistently improved perfusion kinetics ratios of approximately 0.4 (Figure 2E). Finally, by the end of the study, perfusion kinetics ratios for the SKM and hUC hydrogels showed significant improvements over the saline (p < 0.001 for SKM; p < 0.01 for hUC) control, which was maintained at approximately 0.4 (Figure 2F).

**ECM HYDROGELS STIMULATED INCREASED DENSITY OF LARGER ARTERIOLES.** Capillary and arteriole densities were analyzed on tissue cross-sections sampled throughout the entire gracilis muscle at day 35. Although average capillary (Figure 3A) and arteriole density (Figure 3B) did not vary between treatment groups, there was a significant increase in the average arteriole diameter for SKM animals compared to saline (Figure 3C). More specifically there was an increase in the density of larger arterioles with a diameter >75 μm (Figure 3D). Similar trends were observed for the hUC hydrogel, but were not statistically significant compared to saline. A representative image of capillary staining for the SKM group is shown in Figure 3E, while example arteriole staining from all treatment groups is shown in Figures 3F to 3H.

**HYDROGELS WERE FULLY DEGRADED WITHOUT A CHRONIC INFLAMMATORY RESPONSE AND SKM HYDROGEL PROMOTES MUSCLE REMODELING.** On
day 35 post-injection, hindlimb muscle tissue cross sections were stained with hematoxylin and eosin and analyzed by a histopathologist blinded to the treatment groups. Histological analysis concluded that the biomaterials had fully degraded, and no scarring or chronic inflammatory response was observed. Macrophages in the tissue were very low in density, seen as only single cells evenly spaced throughout the tissue. Representative images from the SKM group are shown in Figures 4A and 4B. Next, laminin staining (Figure 4C) was utilized to measure area fraction of ECM to assess interstitial ECM deposition (17). No significant differences were observed with about 20% of the surveyed area being covered by positively stained laminin (Figure 4D).

Fibers with centrally located nuclei can be an indicator of abnormal fibers, representing damaged or remodeling muscle (18). No significant differences in the percentage of fibers with centrally located nuclei were observed by day 35, with an average of about 2% of the fibers (Figure 4E). Individual fiber morphology (cross-sectional area, circularity, and roundness) was also measured and the batched quantified fiber populations were analyzed for animals treated with saline (7,946 fibers), SKM hydrogel (8,302 fibers), and hUC hydrogel (6,000 fibers) compared to healthy tissue (9,130 fibers). The average fiber area of SKM hydrogel–treated animals was not significantly different than the healthy tissue (Figure 4F). In contrast, the average fiber area of the hUC hydrogel group was significantly higher than the SKM hydrogel (p < 0.001) and healthy tissue (p < 0.01), and saline was significantly less than all groups (p < 0.001) (Figure 4F). Next, fiber circularity (ratio of short axis to long axis) and fiber roundness (roughness or angularity) of the muscle fibers also showed SKM was most similar to the healthy tissue (Figures 4G and 4H).

SKM HYDROGEL IMPROVED PERFUSION AND MUSCLE REMODELING THROUGH INCREASED DENSITY OF LARGER ARTERIOLES AND RECRUITMENT OF SKELETAL MUSCLE PROGENITORS. We next sought to analyze the temporal changes following ECM hydrogel injection. We chose to focus subsequent studies on the tissue-specific SKM hydrogel as it had improved muscle remodeling compared to the hUC hydrogel and because its readily available tissue sourcing would make it cheaper and easier to manufacture as a clinical product. We performed an additional hindlimb ischemia study, comparing saline and SKM injections at 3 and 10 days.
post-injection (day 10 and day 17 post-surgery, respectively). There were no significant differences in capillary density, arteriole density, or arteriole diameter between saline and SKM treated muscles at these early time points (Figures 5A to 5F). However, similar to the previous experiment at day 35, density of arterioles >75 mm trended higher in SKM injected animals at both 3 and 10 days post-injection (Figures 5G and 5H).

Due to improvements in muscle remodeling at day 35 post-injection, we analyzed tissue at day 3 and 10 post-injection for infiltration of skeletal muscle progenitors. Pax-7 is a transcription factor involved in early stage muscle lineage commitment in skeletal muscle satellite cells (19). Analysis of percentage of Pax-7 positive nuclei (Figure 6A) showed the impact of SKM injection on the gracilis muscle near the material. At day 3, SKM injected muscles had a significantly higher percentage of Pax-7\(^+\) nuclei compared to saline (Figure 6B) (p < 0.05), but by day 10 Pax-7\(^+\) nuclei dropped to more quiescent levels (Figure 6C).

**SKM HYDROGEL PROMOTES A PRO-REGENERATIVE ENVIRONMENT.** We further investigated the mechanism of action of the SKM hydrogel through whole transcript array analysis. Using a false discovery rate of q < 0.1, there were 561 significantly differentially regulated genes between saline and SKM in the gracilis at 3 days and 16 genes at 10 days (Supplemental Table 5). At 3 days post-injection, Ingenuity pathway analysis showed shifts in the inflammatory response and lipid and carbohydrate metabolism, and increases in muscle development and cell survival with SKM (Table 1). Furthermore, there was down-regulation of genes related to cell death, response to hypoxia, ECM and cytoskeletal organization, and blood vessel development. At 10 days post-injection, there continued to be a shift in the inflammatory response and down-regulation of cell death as well as up-regulation of cell adhesion and motility, ECM organization, blood vessel development, and neural system development pathways. Gene ontology analysis also showed differences in many molecular functions and biological processes, such as binding (GO:0005488), catalytic activity (GO:0003824), metabolic process (GO:0008152), developmental process (GO:0032502), and immune system process (GO:0002376), between groups at both time points (Supplemental Figures 6 to 9). Microarray analysis was validated with quantitative polymerase chain reaction of key genes (Supplemental Figure 3, Supplemental Table 10).

**DISCUSSION**

Various neovascularization therapeutic approaches including cells, growth factors, and gene therapies have been explored, but have been met with mixed results in clinical trials for treating patients with CLI associated with PAD (20). A biomaterial only therapeutic approach could have several advantages compared to the existing paradigm including off-the-shelf availability, reduced cost, and the ability to...
stimulate endogenous cell recruitment over multiple days or weeks. In this study, we demonstrate the first functional analysis of an injectable ECM-based hydrogel for treating PAD by showing increased perfusion in a hindlimb ischemia model and evidence for improvement of the underlying muscle pathology. We also present one of the first studies to investigate the mechanisms of action behind decellularized injectable hydrogels through an unbiased whole transcriptome analysis.

In this study, we used a newer imaging modality, LASCA, to assess perfusion in the feet, which allowed for instantaneous and continuous monitoring of the perfusion in the limbs. This provided insight into the change in perfusion over time. For comparison to previous publications that used laser

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For ages, we show differences in the 2 tissue potential for increased regeneration due to its young approach and as an ECM source with specific ECM composition with human myoblast proliferation(35). When we examined the ischemic muscle shortly after injection of the SKM hydrogel, we found an increase in Pax-7 proliferation and migration, but in vivo, while some perfusion measures showed trends toward an increase with SKM, both materials were capable of significantly enhancing perfusion over the saline control. Decellularized ECMs, including those from xenogeneic sources and those processed into hydrogels, have been shown to promote tissue remodeling and healing in a variety of applications (12,30-32). This positive tissue remodeling has been attributed to a shift toward a pro-remodeling immune response associated with Th2 lymphocytes and M2 macrophages (33,34). Since we observed an increase in perfusion with both materials this could have been caused by this shift toward a more pro-remodeling vs. pro-inflammatory response, and suggests that non-tissue-specific responses such as vascularization may be possible with non-tissue-specific ECM. However, when we examined measures of skeletal muscle remodeling post-ischemic injury, we found that the SKM hydrogel treated animals most closely matched the morphology of healthy skeletal muscle and were significantly different than the hUC hydrogel treated animals. While the 2 ECM hydrogels are derived from different species, they are both xenogeneic in this model. In addition, given the different gelation characteristics, there are likely small differences in the mechanical properties between the 2 gels. However, several studies have shown tissue-specific responses with decellularized ECM in vitro (25-28), many of which used 2D coatings where only the biochemical cues and not the mechanical properties were different. Taken together with our in vivo results, this suggests that tissue specificity of the material may be important for overall tissue remodeling and regeneration. We show through a targeted ECM proteomics approach that SKM and hUC do have distinct ECM component signatures despite being predominantly composed of collagen I. This may be important for muscle regeneration, given evidence in the literature correlating differential ECM composition with human myoblast proliferation (35). When we examined the ischemic muscle shortly after injection of the SKM hydrogel, we found an increase in Pax-7+ skeletal muscle progenitor cells. Pax-7 is up-regulated in regenerating muscle, but usually is expressed in about 5% of nuclei in quiescent muscle (19). This suggests that the SKM hydrogel is causing early stimulation of muscle regeneration through recruitment of satellite cells within the first week of material injection, which may have resulted in the improved measures

Doppler technology, which can only take a static snapshot at a given time point (14,15), typically within 10 min, we reported perfusion measurements at 3 distinct time intervals after the onset of anesthesia. At all 3 readings, the ECM hydrogels significantly increased perfusion over the saline control. In analyzing the equilibrium measurements, which showed the least animal-to-animal variability, perfusion increased over 3 weeks and plateaued at day 21, which corresponds with the material degradation time of approximately 3 weeks (12). The increases persisted out to day 35 post-injection. By using LASCA, we were also able to characterize the dynamic perfusion changes under anesthesia through calculation of the perfusion kinetics ratio. This new measurement provides insight into the health of the vascular network and its ability to transition through a change in cardiac output. By day 35, only the biomaterial treated groups had perfusion kinetics ratios return to 1, indicating the vessel network in the ischemic limb was able to respond to changes in cardiac output at the same rate as the healthy limb. This is clinically relevant because patients with PAD and CLI have shown a decreased or slowed rate of perfusion in transition from states of lower perfusion to higher perfusion. The decreased rate of change of perfusion is associated with decreased vessel health and compliance in diseased patients (21,22). The increase in perfusion and restoration of hindlimb perfusion kinetics directly correlated with histological quantification, which showed an increased density of arterioles with diameters >75 μm throughout the whole muscle, suggesting that the biomaterial therapy acts via promoting arteriogenesis rather than angiogenesis because capillary density was not increased at any of the investigated time points. Other studies have indicated that a therapy impacting arteriogenesis rather than angiogenesis may be more desirable for CLI patients (23,24).

While several in vitro studies have shown the importance of ECM tissue specificity (25-29), limited studies have examined whether tissue specificity is necessary in vivo. In this study, we tested 2 different ECM-derived hydrogels, which were both biocompatible. The SKM hydrogel represents a tissue-specific ECM approach for treating skeletal muscle. For comparison, a new injectable hydrogel derived from human umbilical cords was developed as a non-tissue-specific approach and as an ECM source with potential for increased regeneration due to its young age. In vitro, we show differences in the 2 tissue sources with respect to their effects on cell proliferation and migration, but in vivo, while some perfusion measures showed trends toward an increase with SKM, both materials were capable of significantly enhancing perfusion over the saline control. Decellularized ECMs, including those from xenogeneic sources and those processed into hydrogels, have been shown to promote tissue remodeling and healing in a variety of applications (12,30-32). This positive tissue remodeling has been attributed to a shift toward a pro-remodeling immune response associated with Th2 lymphocytes and M2 macrophages (33,34). Since we observed an increase in perfusion with both materials this could have been caused by this shift toward a more pro-remodeling vs. pro-inflammatory response, and suggests that non-tissue-specific responses such as vascularization may be possible with non-tissue-specific ECM. However, when we examined measures of skeletal muscle remodeling post-ischemic injury, we found that the SKM hydrogel treated animals most closely matched the morphology of healthy skeletal muscle and were significantly different than the hUC hydrogel treated animals. While the 2 ECM hydrogels are derived from different species, they are both xenogeneic in this model. In addition, given the different gelation characteristics, there are likely small differences in the mechanical properties between the 2 gels. However, several studies have shown tissue-specific responses with decellularized ECM in vitro (25-28), many of which used 2D coatings where only the biochemical cues and not the mechanical properties were different. Taken together with our in vivo results, this suggests that tissue specificity of the material may be important for overall tissue remodeling and regeneration. We show through a targeted ECM proteomics approach that SKM and hUC do have distinct ECM component signatures despite being predominantly composed of collagen I. This may be important for muscle regeneration, given evidence in the literature correlating differential ECM composition with human myoblast proliferation (35). When we examined the ischemic muscle shortly after injection of the SKM hydrogel, we found an increase in Pax-7+ skeletal muscle progenitor cells. Pax-7 is up-regulated in regenerating muscle, but usually is expressed in about 5% of nuclei in quiescent muscle (19). This suggests that the SKM hydrogel is causing early stimulation of muscle regeneration through recruitment of satellite cells within the first week of material injection, which may have resulted in the improved measures
of muscle health we observed at 35 days post-injection.

Due to the improvements in muscle remodeling, as well as the improved translational outlook of SKM over hUC because of the ease, cost, and reduced variability with a porcine tissue source, we chose to further investigate dynamic changes in the global transcriptome due to SKM hydrogel injection. Although microarray analysis has previously been employed to characterize the preclinical hindlimb ischemia model (36,37) and human PAD patients (38), no studies have investigated the effect of therapeutic interventions using this global unbiased analysis. At day 3, comparisons between SKM and saline controls showed that the SKM hydrogel promoted cell survival pathways, immune response, and muscle proliferation and contractility, while at day 10 the up-regulated pathways shifted emphasis to vascular development, neuron outgrowth, and cell motility. Genes associated with extracellular matrix (ECM) were down-regulated at day 3 but became up-regulated at day 10. This correlates with other studies of transcriptomic effects of skeletal muscle regeneration, which show that ECM being up-regulated at later time points is associated with tissue repair, cell migration, and myogenic development (39). Interestingly, apoptosis and response to hypoxia were strongly down-regulated at both time points, suggesting a pro-survival and regenerative response of the SKM material compared to the saline control across the entire muscle. These results suggest that the SKM hydrogel produces functional outcomes through altering key pathways associated with inflammatory response, cell death and survival, metabolism, and vessel and muscle development.

CONCLUSIONS

We have shown the efficacy of an injectable biomaterial alone (SKM or hUC) to increase tissue perfusion in a rat hindlimb ischemia study. Significant differences in muscle remodeling at a chronic time point indicate that utilizing a tissue-specific biomaterial therapy for PAD may be more desirable. Furthermore, improved arteriogenesis and skeletal muscle progenitor cell recruitment at shorter time points and gene expression differences related to inflammatory response, blood vessel and muscle tissue development, apoptosis, cell survival, and metabolism give evidence of a transcriptome-wide improvement in tissue regeneration due to the SKM hydrogel.

ACKNOWLEDGMENTS The authors would like to thank Carolina Rodgers and Sandra Leon-Garcia (supervised by Dr. Louise Laurent) for their help with harvesting tissue samples, Matt Joens from the Waitt Advanced Biophotonics Center at the Salk Institute for his expertise with SEM, and Jorge Valencia and Stacey Huynh from the Veterans Association and the Veterans Medical Research Foundation Microarray and Next Generation Sequencing Core at the University of California, San Diego, for their expertise with Affymetrix microarrays.

REPRINT REQUESTS AND CORRESPONDENCE: Dr. Karen L. Christman, Department of Bioengineering, Sanford Consortium for Regenerative Medicine, University of California San Diego, 2880 Torrey Pines Scenic Drive, La Jolla, California 92037. E-mail: christman@eng.ucsd.edu.
COMPETENCY IN MEDICAL KNOWLEDGE: In a rat model, injection of naturally derived ECM hydrogels improved perfusion to the ischemic hindlimb. This is the first pre-clinical study to specifically investigate the effect of ECM hydrogels on functional perfusion in hindlimb ischemia models.

TRANSLATIONAL OUTLOOK 1: Satisfaction of current Good Manufacturing Practice regulations and additional toxicology studies will be required prior to translation; however, this functional study combined with the current clinical use of porcine derived decellularized ECM and initiation of a clinical trial with a similar cardiac ECM derived hydrogel (NCT02305602) provides support for the advancement of the SKM hydrogel into clinical trials for treating patients with advanced PAD.

TRANSLATIONAL OUTLOOK 2: Considering this was a small animal study with a single bolus injection, the optimal number of injection sites and site locations would need to be optimized for human patients. Also, the potential exists for injecting a patient with the therapy several times over numerous months, which was not explored here.


**KEY WORDS** biomaterial, critical limb ischemia, decellularization, hydrogel, injectable, peripheral artery disease

**APPENDIX** For expanded Methods, Results, and References sections as well as supplemental figures and tables, please see the supplemental appendix.
Point-of-Care Technologies for Precision Cardiovascular Care and Clinical Research

National Heart, Lung, and Blood Institute Working Group

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SUMMARY

Point-of-care technologies (POC or POCT) are enabling innovative cardiovascular diagnostics that promise to improve patient care across diverse clinical settings. The National Heart, Lung, and Blood Institute convened a working group to discuss POCT in cardiovascular medicine. The multidisciplinary working group, which included clinicians, scientists, engineers, device manufacturers, regulatory officials, and program staff, reviewed the state of the POCT field; discussed opportunities for POCT to improve cardiovascular care, realize the promise of precision medicine, and advance the clinical research enterprise; and identified barriers facing translation and integration of POCT with existing clinical systems. A POCT development roadmap emerged to guide multidisciplinary teams of biomarker scientists, technologists, health care providers, and clinical trialists as they: 1) formulate needs assessments; 2) define device design specifications; 3) develop component technologies and integrated systems; 4) perform iterative pilot testing; and 5) conduct rigorous prospective clinical testing to ensure that POCT solutions have substantial effects on cardiovascular care. (J Am Coll Cardiol Basic Trans Sci 2016;1:73–86) © 2016 The Authors. Published by Elsevier on behalf of the American College of Cardiology Foundation. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).
The prevention and management of cardiovascular (CV) disease increasingly demands effective diagnostic testing. Consensus defines a diagnostic as a method and an associated device that performs a physical measurement from a patient or associated biological sample and produces a quantitative or descriptive output, known as a biomarker. The definition of a biomarker, in turn, encompasses “a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention” (1). Diagnostics, because of their strategic position at the intersection between patients and their clinically actionable data, directly affect the patient experience and the quality of care that individuals receive (Figure 1). They also furnish valuable tools for clinical investigation. Diagnostics enable providers to improve upon “one-size-fits-all” treatment strategies and instead provide personalized care on the basis of factors such as genetic makeup, comorbidities, real-time serologic assessments, and responses to therapy.

Historically, during the days of “house calls,” diagnostic testing relied primarily on physical examination and bedside analysis of urine (2). As methods for biochemical and cellular biofluid analysis advanced, the portfolio of available tests expanded and central laboratories emerged to standardize sample acquisition and measurement quality while offering economies of scale (3). Today, technology is expanding the number of diagnostic tests that can reach beyond the walls of centralized laboratories and back to the point of care (POC) for use across a broad range of clinical settings. Yet, despite the intuitive appeal of miniaturization and immediate test resulting, point-of-care technologies (POCTs) face important practical questions about their integration into clinical workflows, objective measurement of clinical benefit, standards necessary to ensure quality despite decentralization, and what reimbursement models will engender mutual enthusiasm by payers and providers.

POCTs promise to provide high-quality biomarker measurements optimized for the special constraints of diverse clinical settings including acute care, outpatient clinics, clinical research centers, homes, rural areas, and the developing world (Figure 2). In acute care settings such as the operating room (OR), cardiac catheterization suite, intensive care unit (ICU), or emergency room (ER), physicians seek real-time feedback to optimize care and tailor therapies to the dynamic circumstances they confront. In outpatient clinics, providers look for opportunities to replace reactive medicine with prevention, and to implement “precision medicine,” a national initiative that includes mobile and personal technologies as key components (4). In the home, care teams seek minimally invasive devices that seamlessly integrate health monitoring into daily living. The hope is that longitudinal measurements of home health will supplement episodic clinic visits and transform outpatient care into a data-driven practice. Independent of their health care providers, the public is adopting diverse POC-like self-tracking devices such as sleep monitors, Wi-Fi-connected scales, blood pressure cuffs, finger-stick blood tests, and wearable wristbands and watches linked to cloud storage, analytics, and opportunities for sharing. The degree to which such technologies will improve health care delivery and clinical outcomes remains hotly debated. Ultimately, only rigorous testing will determine their actual clinical utility.

In clinical research, POCTs can expand quantitative data collection to broader populations. By fostering inclusion of under-represented groups in rural areas and the developing world often beyond the reach of traditional clinical trials, POCTs promise to improve the generalizability of study results (5-7).

The National Heart, Lung, and Blood Institute (NHLBI) convened a working group (WG) to examine the translation of CV POCT to precision medicine and clinical research (8). The meeting aimed to provide guidance to the NHLBI regarding the development, evaluation, and dissemination of high-impact POCT in research and treatment. This report summarizes and expands upon the WG discussions by: 1) describing examples of how POCT can address some of the most commonly faced problems in CV disease management; 2) identifying barriers and challenges

Research Institute; has been a speaker for Daiichi Sankyo and Merck; and holds a patent in personalized antiplatelet therapy in interventional cardiology. Dr. Wissleder is a founder of and consultant to T2 Biosystems. Dr. Libby has received research support from the National Heart, Lung, and Blood Institute (R01 HL080472); has served as the Chair of this Working Group; is an unpaid consultant or involved in clinical trials for Amgen, AstraZeneca, Boehringer Ingelheim, Bristol-Myers Squibb, DaCor, Genzyme, GlaxoSmithKline, Kowa, Merck, Novartis, Pfizer, Regeneron-Sanofi, and Takeda; and is a member of the scientific advisory boards for Althea Biotechnologies and Interleukin Genetics. All other authors have reported that they have no relationships relevant to the contents of this paper to disclose. Drs. King and Grazette contributed equally to this work.

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to clinical translation; 3) calling for rigorous clinical testing and validation before integrating new POCTs into routine clinical care; and 4) outlining a POCT development roadmap that articulates specific recommendations to guide NHLBI research priorities.

**IPOC EXAMPLES, CHALLENGES, AND OPPORTUNITIES**

**POCT IN ACUTE CARE SETTINGS.** Practitioners in acute care settings such as the ER, OR, ICU, hemodialysis unit, or cardiac catheterization suite face highly dynamic situations. Real-time POCT promise to improve patient care in these environments by supplying data rapidly to support decision-making, as illustrated in the following examples.

**Example 1: rapid evaluation of ER patients with chest pain—“rule out myocardial infarction”**. In ambulances and ERs, POCT can improve the efficiency of care by enabling rapid assessment and triage of patients with chest discomfort. Cardiac troponin (cTn), a highly sensitive and specific biomarker of myocardial injury, guides triage and management of patients presenting with symptoms suggestive of acute coronary syndrome (9). ERs already use commercial POC cTn assays, but parallel efforts are exploring whether central laboratory cTn assays can perform serial measurements at progressively shorter intervals to discriminate cardiac from noncardiac causes of chest discomfort and enable rapid patient triage. Historically, stable serial measurements of cTn taken at 6- to 12-h intervals served to “rule out” cardiac injury (10,11). More recently, high-sensitivity cTn assays, available only in the central laboratory, permit exclusion of clinically important myocardial injury with high confidence at initial sampling as well as after only 2 serial measurements performed at 1- to 2-h intervals (12–15). POC devices that can match this performance without sending samples to a central laboratory may become mainstream frontline CV diagnostics (Figure 3A).

**Example 2: management of bleeding and clotting risks.** The quandary of balancing the risks of bleeding and clotting concerns practitioners of many specialties. Clot formation involves complex interactions among coagulation factors, platelets, and tissues (16). Surprisingly, a limited number of coagulation diagnostics guide routine outpatient and inpatient management (17,18). Central laboratories typically measure 2 key coagulation parameters: prothrombin time and activated prothromboplastin time. Yet, delays of ~1 h limit the utility of central laboratory measurements for acute care settings such as the ICU or OR, where thrombotic risk can vary moment to moment due to administration of anticoagulant boluses and pharmacological reversal agents (19–21). In these settings, activated clotting time (ACT), a whole blood measurement that integrates intrinsic and extrinsic coagulation with platelet function, commonly serves to quantify thrombotic potential (21). In the case of ACT measurements, procedural technicians, within steps of patient and proceduralist, perform POC testing independent of the central laboratory. This example illustrates the feasibility of integrating real-time POC diagnostics into acute clinical workflow (Figure 3B).

Platelet function complements coagulation in regulating thrombotic risk. Yet, despite extensive studies of platelet function assays in both central laboratory and POC formats, questions remain regarding their incremental benefits. Measures of platelet function do identify populations at higher risk of thrombotic events, but the demonstration that therapy guided by such assays improves outcomes has proven elusive (22–24). This apparent paradox underscores the need to subject any POC diagnostic,
no matter how plausible, to rigorous research to evaluate its efficacy and added value. Coagulation and platelet function biomarkers exhibit variability and context-dependence, adding complexity to their clinical use (25,26). POC diagnostics offer the potential to capture these variations through more frequent measurement, but whether doing so substantively and cost-effectively improves outcomes will require additional research (Figure 4).

Exploratory thrombosis assays aim to complement existing assays of thrombotic risk. Examples include clot relaxation (27) or thromboelastography (16), which evaluate viscoelastic properties of clot formation. Although such assays were initially considered to be too complex for routine clinical use, recent POC adaptations of these measurements aim to improve usability (18,28).

Example 3: future acute care POC assays. Reliable detection of thrombosis presents a diagnostic challenge. Available POC devices can measure thrombosis serum biomarkers such as D-dimer, which notoriously lacks specificity. Often, acute D-dimer elevation due to thrombosis cannot be distinguished from chronic elevation related to comorbid conditions. Instead, modern diagnosis of deep vein thromboses and pulmonary emboli relies primarily on imaging modalities such as ultrasound or contrast chest computed tomography, respectively. Recently, exogenous “synthetic biomarkers” were engineered to supplement endogenous biomarkers and enable more flexible remote monitoring of thrombosis. In concept, an intravascular nanoparticle-conjugated peptide, when cleaved by activated thrombin, liberates a peptide fragment that undergoes renal clearance detectable in the urine centrally or by POC platforms, such as novel paper-based microfluidic assays (29–31) (Figure 5A). Similar synthetic biomarker strategies are under development for a broad range of analytes.

In principle, continuous biomarker monitoring would provide the most complete picture of an individual’s physiological state. Historically, the ability to measure continuously physicochemical biomarkers such as blood pressure, pulse, electrocardiogram, respiration rate, and oxygen saturation revolutionized critical care and substantially improved the safety of general anesthesia. Continuous vital sign measurements have become the standard of care for periprocedural CV monitoring; however, efforts to engineer continuous blood biomarker measurement platforms that monitor “biomolecular vital signs” presents more challenging problems, whose solutions are only nascent (32). The most notable clinically relevant analyte adapted for continuous measurement is glucose. Glucose is readily detected by diverse electrochemical-sensing platforms coupled to an immobilized enzyme (glucose oxidase); yet, the lack of broadly available analyte-enzyme pairs limits the generalizability of this approach. More recently, reversible affinity sensors have promised to expand the portfolio of analytes subject to continuous monitoring. For example, a microfluidic device containing a sensing surface functionalized with nucleic acid-based aptamers can reversibly bind corresponding analytes. The fluidic device directs blood across the planar sensing surface separated by a layer of buffer solution (Figure 5B) such that only biomolecules below a critical
molecular weight can diffuse across the buffer layer and encounter the sensing surface. The reversible binding of analytes to their corresponding immobilized aptamer then generates an electrochemical signal proportional to blood analyte concentration (33). The flexibility of such platforms is expanding as the number of available analyte-aptamer pairs grows. This example also illustrates the exciting potential enabled by integrated microfluidic platforms, which operate at flow rates less than microliters per minute, and can in theory enable continuous blood draws over hours and days while keeping total blood volumes below that of a single conventional blood draw.

POCT IN AMBULATORY SETTINGS. In outpatient clinics, the brevity of the patient visit rather than the dynamics of the physiological state provide the motivation for POC diagnostic testing. Patients frequently have blood drawn for diagnostic testing after a clinic visit. Unfortunately, the ad-hoc follow-up discussions of testing results can lead to undesirable breaks in patient-provider communication. A current movement calling for more diagnostic testing in the clinic aims to resolve such inefficiencies. POC diagnostic platforms in development aim to answer these challenges by enabling measurement of existing biomarkers as well as fundamentally new biomarkers that can enhance the outpatient practitioner’s diagnostic toolkit.

Example 1: miniaturization and mobilization of existing laboratory diagnostics. The earliest examples of POC diagnostics aimed to miniaturize and make portable the measurement of established biomarkers such as the complete blood count or basic metabolic panel. Yet, clinical use of such POC diagnostics has not kept pace with the number of commercially available testing platforms. Instead, there is continued reliance on central laboratories, which likely points to challenges presented by new POC, including cost, uncertain reimbursement, requirements for calibration with legacy central laboratories, standardization of testing procedures, verification of testing expertise, maintenance of the decentralized testing equipment and procurement of disposables, and establishment of good data management practices including security and privacy. Despite these challenges, miniaturized and mobilized versions of existing diagnostic tests, being the first POC diagnostics to enter clinical CV care and research, will likely serve as vehicles for addressing these challenges.

Example 2: personalized CV care using nucleic acid assays. Whether nucleic acid-based assays should be adapted to POC formats remains an area of ongoing investigation. Deoxyribonucleic acid (DNA)-based diagnostics have enjoyed success in oncology because particular DNA mutations inform therapy efficacy. Similarly, in infectious disease, detection of the DNA from an invading pathogen carries clear diagnostic information. Cardiologists, however, have used DNA diagnostics primarily for monogenic conditions. One exception is the assessment of rejection of transplanted hearts, where cell-free (cf) DNA sequences can selectively detect donor heart damage. Indeed, circulating donor cfDNA levels correlate with episodes of acute rejection as determined by invasive endomyocardial biopsy (34,35). Detection of donor cfDNA enabled prospective noninvasive diagnosis of acute rejection with sensitivity and specificity comparable to the biopsy alone (35). A similar cfDNA sequencing approach examined the evolving pathogen landscape in heart transplant recipients in response to changes in their immunosuppressant and antiviral regimens (36). Determining the utility of POC genomic assays for CV transplant rejection or infection assessment will require additional research.

RNA, in contrast to DNA, can change dynamically during disease, making it an attractive biomarker for next-generation CV diagnostics. The complex and multifactorial nature of CV diseases has motivated exploration of transcriptional profiling. One approach uses a 23-gene expression assay platform on the basis
of microfluidics and dehydrated primers, which measures gene expression fingerprints from circulating cells (37,38) to provide negative predictive power to limit the need for more elaborate CV testing. The assay, which in its current instantiation is still far from POC, requires shipment of samples to a central facility. Nonetheless, this early example demonstrates the feasibility of using gene expression fingerprinting as a discriminatory CV biomarker.

**Example 3: next-generation integrated diagnostics platforms.** In addition to soluble proteins and cell-based or cell-free nucleic acids, emerging biomarkers such as rare circulating cells, mRNA, and exosomes, and their contents hold diagnostic promise (39–42). Platforms based on microtechnology and nanotechnology can capture rare analytes from crude patient samples, fractionate specimens, and quantify biomarkers using signal amplification and integrated detection schemes.

Microtechnology uses devices with dimensions on the order of the thickness of a human hair. Built using fabrication techniques originally developed for the microelectronics industry and extended to microelectromechanical systems, these devices are ideally suited for POC handling and analysis of small volumes of complex biological fluid specimens such as blood or urine (43,44). Fabrication in transparent biocompatible polymers renders these devices compatible with conventional optical detectors. Engineers have developed increasingly powerful integrated fluid handling components that now enable dense arrays of highly efficient pumps and valves to precisely control movement of fluids, solutes, and cells (45,46). This powerful toolkit has enabled the design of diagnostic devices that perform a variety of functions, including particle and cell sorting, rare cell capture, and massively parallel and sequential biochemical reactions (41). The design flexibility enabled by microtechnologies offers broad utility for the development of POC devices. Defining clear diagnostic problems in CV medicine that can harness the creativity of this community holds great potential.

Nanoscale devices have a length scale 3 orders of magnitude smaller than that of microfabricated devices. Nanoparticles are key components of these technologies. Among the many ways to detect nanoparticles, strategies on the basis of magnetic properties of the particles or surface plasmon resonance represent particularly elegant examples (47–49). Extension of the size scale of particles can allow sensing by commercially available detectors such as smartphones. For example, in an application requiring counting of cell subsets, investigators bathed biological samples in microbeads conjugated to cell-specific antibodies and used the diffraction pattern of cells decorated with antibody-conjugated beads to identify and count the cell population of interest using a custom dongle attached to a commercially available smartphone (50). Several excellent reviews describe these technologies in further detail and describe examples of additional POC applications (5).
POCT IN THE HOME. Patients spend <1% of their lives interacting with the health care system in the traditional sense. They spend the remaining >99% in the outside world, “at home.” Innovative technologies such as the Internet of Things, wearable devices, mobile communication devices, and social networks promise to improve fundamentally our understanding of human health and transform the home into the next frontier of outpatient medicine.

Outpatient clinical visits frequently begin with open-ended questions such as “have you been taking care of yourself at home?” This question calls on patients to summarize months of post-prandial glucose levels and blood pressure measurements, as well as adherence to recommended exercise, diet, or prescription medications. The availability of more objective and quantitative data can paint a dynamic and unbiased picture of home health across time. Balancing the desire to inform but not overburden providers with extraneous or unactionable information will require thoughtful data synthesis and analytics to traverse efficiently the voluminous data. Third-party disease management businesses may serve as intermediates. Nevertheless, successful navigation of the “big data” problem will transform the home into an informative lens through which one can observe patient health.

The following examples demonstrate the breadth of home health monitoring devices currently available or in development. Together, these technologies promise to create a more comprehensive picture of patient health and behavior that can complement patient self-reporting during in-person health care visits.

Example 1: self-testing POC diagnostics. Blood glucose testing remains one of the oldest and most widely accepted POC applications. Yet, despite substantial investment and widespread use, few sufficiently powered studies have examined the clinical utility and cost-effectiveness of glucose self-monitoring (51,52). Meta-analyses estimate glycated hemoglobin declines of 0.22% to 0.40% in patients using blood glucose self-monitoring compared with control subjects (52,53). Although these studies did not directly evaluate the effect on clinical outcome, this magnitude of reduction associates with a significantly reduced risk of microvascular complications in other clinical trials (54). Studies that involved the inclusion of glucose self-monitoring as a component of a structured therapeutic management program, including education and follow-up, yielded the greatest improvements in outcomes (55).

Monitoring of anticoagulation in warfarin-treated individuals with POC international normalized ratio measurements furnishes another example of self-testing. A meta-analysis of 11 randomized controlled trials showed significant reductions in the risk for thromboembolic events (hazard ratio: 0.51; 95% confidence interval [CI]: 0.31 to 0.85), with no increase in major hemorrhagic events or death (hazard ratios: 0.88; 95% CI: 0.74 to 1.06; and 0.82; 95% CI: 0.62 to 1.09, respectively) in patients who self-monitored compared with patients who did not (56). In addition, the coupling of self-monitoring with self-management and dosing was associated with greater risk reductions. Thus, although self-monitoring of the international normalized ratio may not benefit all patients, wider access and availability of testing in the home can strengthen the effectiveness of care.

Example 2: connected diagnostics—wearables, smart phones, and the “Internet of Things.” Wearables. The ability to position sensors onto a patient and into the clothing they wear has powerful potential to transform our understanding of CV health and disease. These categories of POC devices, sometimes termed “wearables,” a subset of the “internet of things,” the “Internet of Things,” have captured the imagination of physicians and patients alike. The earliest versions of internet-connected home health devices simply adapted conventional diagnostics previously used in the clinic or hospital, such as blood pressure cuffs, heart rate monitors, scales, pedometers, oximeters, and positive pressure ventilation controllers. Telehealth programs and chronic disease management practices developed systems to monitor data from these sensors, provide feedback regarding results to providers and/or patients, and encourage compliance using reminders. More recently, wearable technologies such as wristbands and watches equipped with integrated microscale accelerometers have enabled activity monitoring, performance feedback, and the ability to annotate data with subjective measures of health for sharing with friends, family, health care providers, or the world.

High-performance electronic circuits play an essential role in home health devices, but rigid circuit boards and wires present a barrier to miniaturization and integration into ambulatory-sensing solutions. The recent development of a flexible electronics platform permits the fabrication of high-functioning electronic devices into thin flexible tattoo-like transparent films that adhere to the skin (57,58,63,64). The durability of initial prototypes underwent testing in challenging locations such as the elbow without signal degradation over days despite repeated extension and flexion. Transmission of the electrocardiogram and electroencephalogram...
confirmed the functional utility of the device. Optimization of such technologies should permit continuous remote measurements in outpatients. Monitoring of atrial fibrillation could benefit from such advances. The burden of atrial fibrillation and the frequency of atrial fibrillation paroxysms likely correlate with stroke risk (59,60). The emergence of minimally invasive heart rate and rhythm-monitoring devices on the basis of flexible electronics and other technologies offers a unique opportunity to document longitudinally patient rhythms in relation to other life events (61). Such information would enhance our understanding of triggers associated with arrhythmia onset and termination and aid patient management.

Internet of Things. A movement termed the “Internet of Things” aims to convert the home into a densely interconnected environment with embedded sensors in everyday objects that can monitor, communicate, and connect the environments in which we live. As an example of this technology, bedroom-embedded sleep monitors aim to optimize rest and detect sleep-disturbed breathing. The embedded sensing concept, although still in its earliest stages, has excited CV care providers with the possibility of devices that will monitor high-risk patients, identify early warning signs of decline, and prompt early intervention that may avoid more severe decompensation.

Management of heart failure is particularly poised to benefit from emerging home health technologies (62). Heart failure affects more than 5 million U.S. patients, triggers >1 million hospitalizations annually, and associates with remarkably high rehospitalization rates (~25% at 30 days and ~50% by 6 months) (63). Because weight gain often precedes hospitalization by days to weeks, some guidelines recommend that patients weigh themselves daily at home. Unfortunately, adherence to this recommendation remains poor; in a recent large-scale clinical trial, compliance with telemonitoring fell from 90% to 55% by 6 months despite implementation of an aggressive reminder system (64). This deficiency challenges disease management teams and practitioners caring for patients at home. Hence, a need exists to develop devices that monitor activity and/or weight without proactive patient participation. Doing so should improve the regularity of home testing and may avert unnecessary hospitalizations through early detection of volume overload (Figure 6).

Medication compliance also received early attention. Adherence to recommended pharmacological therapy remains an important but often unappreciated challenge of outpatient CV care. This challenge spurred the development of Wi-Fi-connected pill bottle caps and internet-connected sealable blister packs, inhalers, or injectables to provide new windows into patient medication compliance. This capability enables study of whether incentivization strategies and gamification can improve adherence to daily medications such as statins. Although recording patient medication access times does not directly reveal ingestion, these data nevertheless provide previously unobtainable information about patient medication habits at home. To measure more directly medication adherence, developers have created a microchip sensor-enabled pill with Food and Drug

**FIGURE 6** Management of Patients With Heart Failure Will Benefit Tremendously From POCT in the Home

This patient population is benefiting from new point-of-care technologies (POCTs) that longitudinally monitor biomarkers of heart failure decompensation (e.g., symptoms, weight, and ventricular filling pressures) to guide adjustments in diuretic dosing and avoid unnecessary hospitalizations. BBQ = barbecue, a high-salt meal.
Administration (FDA) clearance that communicates with an adhesive patch worn on the torso that records when the pill is ingested (65). Early studies using this technology reportedly suggest that patients with greater irregularity in the timing of their morning medication were more likely to miss doses altogether and had lower medication adherence rates across time. This result suggested that interventions, such as incorporating therapy into a different facet of a daily routine, might improve compliance. Combining integrated sensing technologies such as these with behavioral studies is a fertile area for future research.

Smartphones. Smartphones serve as powerful platforms for software and hardware developers to collect, store, manage, and communicate health sensor data. In the CV space, a smartphone case with integrated contact electrodes allows a user to measure continuously an electrocardiographic rhythm strip simply by holding the case with 2 hands. Combined with rhythm detection software and the ability to save and share tracings, this technology has the potential to expand greatly patient self-recording of single-lead heart rhythms. For diabetic individuals, an electrochemical blood glucose meter that attaches to commercial smartphones as a dongle can measure, store, and analyze glycemic control. Similarly, full laboratory-quality immunoassays have also been miniaturized and adapted to a custom dongle attached to a commercial smartphone (66).

Smartphones have generated tremendous excitement for use in clinical research. Apple’s ResearchKit (Apple, Cupertino, California), which was downloaded with great enthusiasm upon its initial release, allows users to participate in clinical research via their smartphones and iPads. Applications focusing on CV disease (MyHeart Counts) and diabetes management (GlucoSuccess) were among the initial offerings and feature the ability to monitor activity, self-record a 6-min walk test, and record dietary habits and medication adherence. Google X, Duke, and Stanford also recently announced an ambitious project, the Baseline Study, which aims to understand what keeps people healthy and what determines disease trajectories. As the pilot phase of the study gives way to larger cohorts, reports suggest that the study will collect genomic information and employ more complex human phenotyping from the “Study Kit” application, associated devices, and even wearables such as the much anticipated “smart” contact lenses. These innovations extend the POC concept to everyday life and provide enormous potential for mining “big data” for health purposes at a population level, but also enabling precision medicine or personalized management for the individual.

Example 3: invasive outpatient health monitors. Implantable monitors are inherently invasive and, therefore, require careful consideration of safety before use. Once placed, however, this class of monitoring devices makes several potentially powerful biomarkers available to providers longitudinally across time. For example, implantable rhythm recorders as well as conventional pacemakers and implantable cardiac-defibrillators can detect rare but concerning paroxysmal ventricular arrhythmias as well as exhaustively profile the timing and burden of chronic arrhythmias such as atrial fibrillation. An ambulatory intrathoracic impedance monitor and associated algorithm attempt to identify thresholds and temporal signatures of impedance changes that predict worsening heart failure (67-69). Another device still in development directly measures left ventricle filling pressures in the left atrial appendage, but requires transeptal puncture for device placement. The data, which are transmitted to a hand-held patient advisory tool, can then guide medication dosing changes according to an algorithm. Taking a different approach, a new FDA-approved implantable pulmonary artery pressure monitor can be placed during a right heart catheterization and does not require a transeptal puncture. In the COMPASS-HF (Chronicle Offers Management to Patients With Advanced Signs and Symptoms of Heart Failure) trial that evaluated this technology, increases in PA pressure were reportedly more sensitive and specific, and they anticipated weight increase associated with decompensation (70,71). Support for device approval largely stems from the CHAMPION (CardioMEMS Heart Sensor Allows Monitoring of Pressure to Improve Outcomes in NYHA Class III Heart Failure Patients) trial, which demonstrated that in New York Heart Association functional class III heart failure patients hospitalized in the past 12 months, management guided by the pulmonary artery pressure monitor significantly reduced heart failure admissions (72).

PRACTICAL ASPECTS OF POCT IMPLEMENTATION

CLINICAL TESTING AND VALIDATION OF POCT. POCT development must balance the enthusiasm for promising new diagnostic platforms with the need for rigorous validation studies. Adoption of a new device should depend on demonstrated performance compared with reference standards of care to ensure
that it provides similar or improved clinical utility. Even if POC diagnostics cannot demonstrably alter hard outcomes such as survival, they may provide added value by limiting lengths of stay, reducing readmissions, avoiding unnecessary invasive tests, boosting physician and/or patient satisfaction, improving quality of life, or benefiting other aspects of health care delivery, cost, or comparable metrics. The success or failure of a POC diagnostic depends critically on establishing clearly stated goals and conducting rigorous research to evaluate its ability to meet pre-specified objectives. Anticipating potential risks of POC has equal importance. For example, more diagnostic availability could conceivably increase testing volume and cost. More testing may also trigger more false positive results and lead to more invasive downstream testing, which would needlessly alarm patients and practitioners alike. Research that addresses such health systems and patient-provider communication issues associated with POC could vitally and meaningfully affect patient care.

**ETHICAL AND REGULATORY CONSIDERATIONS.** Although POC testing offers many advantages as a clinical tool, decentralization of diagnostic testing may require new regulations to maintain procedural standardization, adherence to calibration standards, and maintenance of patient privacy. The FDA, in its oversight role, has provided related guidance such as the “regulatory oversight framework for laboratory developed tests” and the “mobile medical application policy” (73,74). Practitioners should also be aware that under current regulatory requirements, POC devices are not necessarily waived under CLIA (Clinical Laboratory Improvement Amendments) simply because of use at the point of care. The use of POC devices for clinical research requires development of a policy regarding sharing of results with participants. Protecting the health information of patients remains fundamental to clinical care by ethics and statute. The design, ownership, and operation of new POC devices requires cooperation by multiple partners to capitalize on the data collected without compromising patient privacy. These concerns mirror those currently encountered with widespread adoption of electronic health records. Yet, the mobile nature of POC devices decentralizes privacy preservation and entrusts sensitive patient data to a broad range of individuals spanning health care specialists to POC data management service providers. The active research of the HHS suggests the need to update provisions of the Health Insurance Portability and Accountability Act of 1996, now influenced by challenges related to the rapid developments of health information technology, including implementation of electronic medical records and mobile technologies for health care (75).

**POCT AS A CLINICAL RESEARCH TOOL**

POCT have considerable potential to enhance clinical research. Biomarkers can contribute to clinical endpoints by complementing clinical endpoints with interim measurements that can deepen understanding of interventions. In this context, POC diagnostics offer powerful opportunities to enrich biomarker collection during the conduct of well-controlled clinical trials with carefully adjudicated endpoints. Ambulatory monitoring devices such as mobile device applications, wearable monitors, and home-based sensors remain some of the most attractive POC categories as aids to clinical investigation. Unlocking the vast assortment of uncaptured ambulatory data presents an opportunity for POC devices to enrich substantially clinical trial data collection. Similarly, biofluid sampling during clinical trials remains a valuable resource when paired with carefully curated patient populations meeting well-defined entry criteria that can correlate with carefully adjudicated endpoints. Yet, limits pertain to the number of biofluid biomarkers that conventional assays can measure. POC technologies that seek to multiplex biomarker measurements have a tremendous potential to maximize the information yield from these scarce samples and facilitate biomarker discovery, biomarker validation, and mechanistic insight.

Additional benefits of POC in clinical research include enabling novel patient recruitment pathways by facilitating screening for eligibility criteria without requiring centralized testing or return visits. POCTs also offer flexible pathways for baseline and follow-up data collection, providing opportunities to improve clinical trial quality control through longitudinal and site-specific monitoring of study protocol compliance. POC diagnostics could expand opportunities for clinical trial participation beyond heavily populated urban areas, where trial coordination is typically centered. Doing so will reduce the inherent selection bias associated with existing geographic constraints and make trial results more relevant to a broader population. The National Institutes of Health has initiated funding for several clinical trials to test such technologies; continued development of this approach will represent an important advance in CV clinical research (see Table 1 for examples).

Clinical research in the developing world often focuses on communicable diseases; however, CV disease remains the leading cause of death worldwide and does not spare low- and middle-income
POCTs have tremendous potential to advance CV care. Realizing this potential will require a funding environment that incentivizes engineering solutions beyond mere proof of concept demonstrations and cultivates them throughout the full technology development life cycle. Through close collaboration, engineers and health care providers must work together to ensure that innovative POC devices have a path for clinical testing, regularly undergo quantitative comparison to modern standards of care, and require careful monitoring to achieve POC goals without making excessive performance compromises. The NHLBI occupies an ideal position to incentivize formation of multidisciplinary teams comprised of health care providers, biomarker scientists, technologists, and clinical trialists to collaborate longitudinally throughout the development process. To guide the activities of these CV POCT teams, the NHLBI WG proposes the following 5-stage CV POCT Development Roadmap. This approach aims to capitalize on advances in biomarker science and emerging sensor technologies to create clinically relevant POC devices with a defined path for rigorous clinical testing and validation.

1. Needs identification. Stage 1 goals include the identification of specific aspects of clinical care that have potential for substantial improvement by POCT “clinical needs” and the articulation of (well-posed) clinical problems that carefully describe the process of testing a specific POCT solution (which patient population, what clinical setting, the methods behind the quantitative measurement of benefits and risks, and the standard[s] of care that will serve as comparison).

2. Biomarker selection and device design specification. Stage 2 will bring biomarker scientists and technologists together to take the “well-posed clinical problem” from stage 1 and use it to both select a relevant set of new and/or old biomarker(s) and define design criteria for a corresponding POC measurement device. Defining the clinical objectives early increases the likelihood of success once the technology exists.

3. Device development. Stage 3 will focus on development of component technologies and system integration, leading to a functional POC measurement device. Guided by the specifications defined in stage 2, an iterative process of design, fabrication, and testing of key technologies and individual components will be undertaken. This will culminate with a system integration process that will require additional engineering and performance characterization using simplified models of the relevant human biomarkers.

4. Pilot testing. Stage 4 will use the POC solution developed in stage 3 to perform pilot testing on clinical samples or small-scale patient populations. This effort will help identify obstacles associated with real-world biosamples, identify normal ranges and intervention thresholds, highlight data management issues, reveal unanticipated human factors, and provide initial data on device usability in a real-world clinical setting. An iterative process of device refinement and repeat pilot testing will prepare the technology for rigorous clinical testing in stage 5.

5. Prospective clinical testing. Stage 5 will test and validate the technology in “real-world” health care settings. Assessment of device efficacy as well as liabilities and risks remains the overall goal. Because design tradeoffs are device- and application-specific, the criteria for success will need to be defined on a case-by-case basis (defined during stages 1 and 2). Acceptance of methodologies and validation studies for all new diagnostics including POC should require transparency and peer-reviewed publication.

RECOMMENDATIONS FOR NHLBI CV POCT PRIORITIES

- Support Stage 1 activities with the engagement of the broad CV and bioengineering communities in pre-competitive CV POCT needs-assessment activities. This action would ideally extend beyond a
single meeting to include the creation of a forum for an ongoing electronic and living discussion that encourages broad participation from diverse backgrounds and spans multiple training levels. A multidisciplinary team of experts can consolidate, curate, and refine contributions to create a focused list of Cardiovascular POCT Grand Challenges that correspond to the most promising “well-posed clinical problems.” The most attractive Challenges will ideally extend beyond modest reductions in assay sample volume or increased portability of existing biomarker measurements, and instead pursue ambitious goals that ensure that novel CV POCT can improve dramatically CV care. Challenges that offer an opportunity for the CV POCT community to coalesce around a small number of high-impact problems are most likely to inspire development of high impact technologies.

- Support stage 2 activities with the funding of biomarker science (basic discovery and validation of novel CV biomarkers) and novel sensing technologies (methods for detecting and quantifying a variety of biomarkers from diverse patient-derived samples). This fundamental work will cut across individual clinical POC devices and provide a scientific and technological basis for integrated POC solutions.

- Support stage 3 activities with the funding of specific implementations of biomarker-sensing solutions that range from individual sensing components to complex, fully integrated POC sensing platforms that perform “sample-to-answer” measurements from clinically relevant specimens.

- Support stage 4 activities by enabling pilot testing of integrated “sample-to-result” technologies developed in stage 3 via small-scale, proof-of-concept studies in preparation for larger-scale prospective POC testing in stage 5. Support could include facilitating access to annotated clinical specimens. Such pilot testing can also help determine reference values for POC tests in relevant populations, encourage harmonized data standards and reporting of POC tests, and determine the data and technical integration needs at different levels (e.g., technical, systems, patient data, and population).

- Support stages 4 and 5 with core data storage, data processing, privacy, and analytics that will permit the transmittance of POC results to and from patients and providers to realize their power without compromising patient privacy.

- Integrate stage 4 and 5 POCT activities with ongoing or planned clinical studies funded by the NHLBI using funding mechanisms such as ancillary studies or Small Business Innovation Research/Small Business Technology Transfer grants, and make existing clinical data and relevant biobanks accessible to aid validation of POC technologies against established centralized laboratory measurements.

- Assess standards for evaluation and clinical use of POC tests through the establishment of application-specific standards of quality, efficiency, affordability, accessibility, and safety of devices and data (in coordination with other appropriate agencies.)

- Fund career development activities, program formation, and industry surrounding novel biomarker science and POC technology development that provides the foundation for new POCT opportunities on the basis of previously unrecognized or unmeasurable biomarkers.

POCT possess the potential to transform medical research and patient care. Implementation of a research and development program as proposed in the roadmap and recommendations that emerged from this WG can accelerate the realization of this promise.

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**KEY WORDS** biomarkers, clinical trials, precision medicine
Temporal Trends and Factors Associated With Cardiovascular Drug Development, 1990 to 2012

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SUMMARY
Cardiovascular disease remains a leading cause of death, but stakeholders have recently raised concerns about the pace of innovation and investment in developing new therapeutics. Here, the authors characterized temporal trends in cardiovascular research and development over the past 2 decades and the likelihood of successful completion of preapproval clinical trials. The authors also evaluated the reasons for discontinuation, novelty, and rates of trial results publication for cardiovascular therapies in late-stage development. Between 1990 and 2012, the number of cardiovascular drugs entering clinical trials declined across all stages of development (p < 0.001 for linear trends). There was no evidence for a difference in probability of successful progression to the next stage of development between cardiovascular and noncardiovascular drugs. Small and medium-sized companies sponsored 43%, 38%, and 31% of new Phase 1, Phase 2, and Phase 3 trials, respectively. Roughly one-half of the drugs in Phase 3 trials were categorized as targeting a novel biological pathway. The number of cardiovascular trials sponsored by small and medium-sized companies and the number of novel drugs entering Phase 3 trials increased over time. Most drugs were discontinued in Phase 3 due to inadequate efficacy (44%) or safety issues (24%), but the Phase 3 trial results for only one-half of the discontinued drugs were published in peer-reviewed journals. These results shed light on important shifts in research and development activity and confirm the perceived challenges in cardiovascular translational research. (J Am Coll Cardiol Basic Trans Science 2016;1:301–8) © 2016 The Authors. Published by Elsevier on behalf of the American College of Cardiology Foundation. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

The development of new prescription drugs and their adoption into clinical practice have been associated with significant reductions in cardiovascular mortality over the past 2 decades (1). Despite this progress, cardiovascular disease is a leading cause of death in the developing world and still accounts for 1 in 3 deaths in the United States (1-5). The productivity of translational research in this field has recently come under scrutiny amidst concerns over the declining pipeline of novel therapies (6). Proposed explanations for the discrepancy between the slowdown in innovation and burden of disease include the rising cost of conducting large cardiovascular outcome trials, stagnating financial investment, and diminished commercial attractiveness of the cardiovascular field owing to availability of
low-cost generic medications (6,7). Several high-profile failures of clinical development have contributed to this perception. For example, in 2012, a large Phase 3 trial of varespladib, a secretory phospholipase A2 inhibitor hypothesized to improve cardiovascular outcomes, was halted when an interim analysis found that the drug was in fact associated with an increased risk of myocardial infarction (8).

There are limited data on trends in cardiovascular research and development and the factors associated with the success of new therapies in clinical trials. It has been previously reported that the number of new cardiovascular drugs approved by the U.S. Food and Drug Administration (FDA) has declined in recent years (6,9). A contraction in the pool of cardiovascular drugs under development has also been reported (10), but trends in new drugs that have entered clinical testing or those that have been discontinued remain undefined.

In this study, we describe temporal trends in cardiovascular drug development over the past 2 decades, analyze the likelihood that investigational cardiovascular drugs successfully complete pre-approval clinical trials, and characterize the novelty of drug pathways, reasons for discontinuation, and rates of publishing trial results for new drugs in late-stage development.

METHODS

DATA SOURCES AND EXTRACTION. We analyzed data from a large commercial database of drug development activity (Citeline Pharmaprojects, Informa plc, London, United Kingdom), which tracks in real time the pipeline of pharmaceutical research and development projects. This database covers more than 50,000 products for all diseases from pre-clinical to commercialization stage and is widely used by industry and researchers to analyze trends in drug development (11-15). Using methods described previously (16), we selected for analysis all products that had entered Phase 1 clinical trials between January 1, 1990, and December 31, 2012 (N = 4,715). For each product, we extracted key information, including generic and proprietary names, sponsor, primary indication, mechanism of action (if known), start and end dates of each phase of clinical testing, date of regulatory approval (if applicable), and date and reason for discontinuation (e.g., failure to demonstrate efficacy, safety concerns, commercial/financial).

On the basis of the primary indication, each product was mapped to an Anatomical Therapeutic Chemical (ATC) code, which categorizes drugs according to the organ or system on which they act and their therapeutic and chemical characteristics. We focused on drugs intended to treat disorders of the cardiovascular system (ATC code C), such as antihypertensive, antiarrhythmic, antiarrhythmic, antianginal, and lipid-lowering agents, and disorders of blood and blood-forming organs (ATC code B), such as blood fraction and plasma substitutes, and anticoagulant, antithrombotic, anti-fibrinolytic and antianemic agents. We also compared rates of cardiovascular drugs entering clinical trials with those of cancer drugs (ATC code L01) and central nervous system (CNS) drugs (ATC code N, except N01 and N02) (11). We categorized all sponsors in our study cohort into large pharmaceutical companies, defined as companies with gross revenues >$1 billion, and small and medium-sized companies. Next, we searched Medline, EMBASE, and Web of Science for peer-reviewed publications of trial results, and search engines, press releases, and other publicly available sources for the stated reasons (if any) for discontinuation of drug development.

Finally, 2 investigators (T.J.H. and J.C.L.) categorized cardiovascular drugs that entered Phase 3 trials during our study period as targeting a “novel pathway” or “other target.” Consistent with prior studies by the FDA and others (17-19), we defined a novel pathway as a target or biological pathway for which the FDA had not yet approved a therapeutic agent by the pivotal trial start year. Changes in formulation (e.g., the first oral alternative to existing intravenously administered products) and new combinations of existing drugs (with or without a new agent) were considered novel pathways. Changes in chirality (e.g., a purified single enantiomer form of an already-approved racemic drug) were not considered to be a novel pathway. Any disagreements (representing ~5% of cases) were resolved by consensus.

All data were initially downloaded on June 28, 2013, and information on publication status and novelty was updated through March 1, 2016. This study was not submitted for institutional review board review, because it is based on publicly available data and involved no patient health records.

OUTCOME MEASURES. We first studied temporal trends in the number of new Phase 1, 2, and 3 clinical trials started for investigational cardiovascular drugs over time and compared these trends to those for drugs intended to treat cancer and CNS disorders. We also evaluated the proportion of such trials started by small and medium-sized companies. Because the
The absolute number of new trials would not capture differential rates of development activity among therapeutic areas, the primary outcome was the proportion of new cardiovascular drug trials relative to all new clinical trials in a given year.

Our second outcome of interest was the likelihood of survival of cardiovascular drugs, defined as the probability of successfully proceeding from one clinical trial phase to the subsequent phase of development (e.g., Phase 1 to Phase 2). Because companies often discontinue development projects without public disclosure, we used a conservative approach to identify “implicitly” discontinued products by assuming that projects with no development reported for 3 calendar years or more from the start date for Phase 1 and 5 calendar years or more for Phase 2 and Phase 3 were discontinued. Our third outcome of interest was the proportion of new Phase 3 trials started for drugs targeting novel pathways over time. Finally, we analyzed the reasons for discontinuation of cardiovascular drugs in late-stage development (i.e., during or after Phase 3 trials) and the rate of publication of the results from these trials.

**Statistical Analysis.** We calculated the proportion of new Phase 1, 2, and 3 clinical trials for cardiovascular drugs relative to all new clinical trials and compared to those for cancer and CNS drugs, the proportion of new trials started by small and medium-sized companies, and the proportion of Phase 3 trials started for drugs targeting novel pathways. We used linear regression for trend analysis of continuous variables, and we used the Fisher exact test to compare the difference in proportion of new trials started by small and medium-sized companies to cardiovascular versus noncardiovascular drugs.

To determine the probability of progression of drugs in development, we constructed Cox proportional hazards regression models for each phase change (i.e., Phase 1 to 2, Phase 2 to 3, and Phase 3 to regulatory filing). A key assumption of the Cox proportional hazards model is the proportionality of hazards. Although no violations of proportionality were observed in the Phase 3 model, the assumption of proportional hazards was not met for the Phase 1 and Phase 2 models, indicating that the estimated hazard ratios (HRs) from these models should be interpreted as the average HR over time. For the models corresponding to progression from Phase 2 to Phase 3 and from Phase 3 to regulatory filing, we restricted our analysis to products that entered testing by January 1, 2008, because inclusion of more recent trials may bias our results due to our 5-year discontinuation threshold. As a sensitivity analysis, we used logistic regression and also repeated our analysis excluding hematologic drugs (i.e., only ATC code C).

Next, we constructed multivariable linear regression to examine factors associated with drugs categorized as targeting novel pathways. Models included all variables of interest regardless of statistical significance: firm type (an indicator variable for large pharmaceutical company vs. small and medium-sized companies), indicators for therapeutic area, and a continuous time variable. Finally, we used descriptive statistics to summarize the reasons for cardiovascular drug discontinuations as due to efficacy, safety, or commercial or other reasons.

Statistical analyses were performed using Stata version 12 (StataCorp, College Station, Texas). Two-tailed p values \(<0.05\) were considered statistically significant.

**Results.** Our study cohort comprised 347 cardiovascular drugs that entered Phase 1 testing between 1990 and 2012, of which 239 (69%) were categorized under ATC code C, corresponding to drugs for the cardiovascular system (e.g., antihypertensive agents) and 108 (31%) were categorized under ATC code B, corresponding to hematologic drugs (e.g., antithrombotic agents and blood substitutes). The most common types of products under development were antihypertensive agents (18%, 19%, and 25% of new cardiovascular Phase 1, 2, and 3 trials, respectively), lipid-lowering agents (22%, 20%, and 12%), and anticoagulants (9%, 6%, and 9%).

The number of new cardiovascular drugs entering clinical trials in all stages of development declined over time (p \(<0.001\) for linear trend in Phases 1 to 3) (Figure 1). Between 1990 and 1995, 108 of 679 (16%) Phase 1 trials were initiated for cardiovascular drugs, compared with 125 of 2,366 (5%) Phase 1 trials between 2005 and 2012. Similarly, cardiovascular drugs accounted for 21% of all Phase 3 trials in 1990 but only 7% in 2012. This decline was similar to that for CNS drugs, whereas the number of new cancer drugs increased over the same period (Figure 2).

Large pharmaceutical companies sponsored most clinical trials for investigational cardiovascular drugs. Overall, small and medium-sized companies accounted for 43% of new Phase 1 trials for cardiovascular drugs, 38% of new Phase 2 trials, and 31% of new Phase 3 trials. In all 3 phases, the proportion of cardiovascular trials started by small and medium-sized companies was significantly smaller than that for noncardiovascular trials (p \(<0.001\) for all phases), but the number of cardiovascular trials sponsored by...
small and medium-sized companies grew over time (Supplemental Figure 1).

**PROGRESS OF CARDIOVASCULAR DRUGS THROUGH DEVELOPMENT.** We found no evidence for a difference in probability of survival (defined as successful progression to the next stage of development) between cardiovascular and noncardiovascular drugs in Phase 1 (HR: 1.13; 95% confidence interval [CI]: 0.78 to 1.38; \( p = 0.41 \)), Phase 2 (HR: 0.74; 95% CI: 0.05 to 1.18; \( p = 0.29 \)), and Phase 3 (HR: 1.09; 95% CI: 0.54 to 1.43; \( p = 0.70 \)) (Figure 3). In a sensitivity analysis excluding hematologic drugs (ATC code B), similar results were obtained, with no significant differences in probability observed between cardiovascular and noncardiovascular drugs in all phases of development (\( p > 0.06 \)). Repeating our analysis with logistic regression yielded substantively similar results.

**DRUGS TARGETING NOVEL BIOLOGICAL PATHWAYS.** Overall, 50% (89 of 177) of cardiovascular drugs entering Phase 3 trials were categorized as targeting a novel biological pathway. Over time, the rate of novel drugs entering Phase 3 trials increased, with novel drugs accounting for 27% of Phase 3 cardiovascular trials in 1990 to 1991 and 57% in 2012 (\( p = 0.004 \) for linear trend) (Supplemental Figure 2). In multivariable analyses, none of the studied variables were significant predictors of novel drug status.

**DISCONTINUATION OF DEVELOPMENT FOR EFFICACY AND SAFETY REASONS.** Among 63 cardiovascular drug discontinuations in Phase 3, the reasons for discontinuing development were identifiable for 54 (86%) (Figure 4). Clinical development for 28 (44%) cardiovascular drugs was discontinued due to poor efficacy, and 15 (24%) were discontinued due to safety issues, including 7 drugs that were associated with an increased risk of death. A further 11 (17%) drugs were discontinued for commercial or other strategic reasons. The results from the Phase 3 trials were published in peer-reviewed journals for roughly one-half (32 of 63, 51%) of these drugs with discontinued development.

Selected examples of discontinuations of cardiovascular drugs due to efficacy or safety reasons are shown in Table 1 (20–23). For example, the development of several lipid-modulating agents was discontinued after disappointing results from large controlled trials. A Phase 3 trial of torcetrapib, a cholesteryl ester transfer protein (CETP) inhibitor, was prematurely terminated in 2006 after a significantly increased risk of death was observed in treated patients (23). Nolomirole, a neurohormonal agent and selective dopaminergic \( D_1 \) and adrenergic \( \alpha_2 \) agonist, failed to show benefit in reducing or slowing time to death or hospitalization among patients with heart failure (21).

**DISCUSSION**

This study sheds light on several important shifts in cardiovascular research and development activity that have occurred over the past 2 decades. We found that the share of new cardiovascular drugs entering...
clinical trials has fallen since 1990, both in absolute terms and in comparison to drugs in other therapeutic areas, such as the development of new cancer therapeutics. In parallel, over time, small and medium-sized companies have sponsored a greater proportion of trials for new therapies.

Our findings confirm the perceived challenges in cardiovascular translational research, extending previously reported declines in successful cardiovascular drug approvals (6) to show that there have been fewer new investigational cardiovascular drugs across all stages of clinical development, particularly in late-stage development. Although the numbers may be declining overall, we also found a relative growth in the number of drugs entering late-stage testing that targeted novel biological pathways, suggesting that the observed contraction in cardiovascular research output may be driven by fewer follow-on drugs. This trend is consistent with a prior study that found a decrease over the past decade in the number of FDA approvals for drugs that were not first-in-class (17).

Cardiovascular drugs do not appear to be any less likely to successfully complete clinical trials than other drugs, even in Phase 3. Although they can be time consuming and costly to conduct, Phase 3 comparative clinical trials also provide the highest level of evidence on safety and efficacy to justify utilization in the intended patient population. As such, the consensus of stakeholders from industry, academia, and regulators was that “despite a temptation to use surrogate endpoints to decrease sample sizes and shorten the duration of clinical trials (ostensibly to reduce the likelihood of drug-development failures)[...we must continue to promote large, pragmatic trials to measure clinical outcomes when evaluating new cardiovascular therapies” (6). For example, a recent FDA advisory committee stressed that low-density lipoprotein (LDL) cholesterol levels were not a reliable surrogate for cardiovascular benefit, and that timely completion of outcome trials was imperative (24). Among cardiovascular drugs for which the development was halted in Phase 3, our study found that most were discontinued due to inadequate efficacy, underscoring the importance of rigorous trials in elucidating the risk–benefit balance of new treatments.

In some cases, early and compelling evidence of superior efficacy, compared with existing treatment options, tilts the risk–benefit balance towards faster patient access, and various regulatory programs can be leveraged to appropriately expedite the development and approval of these treatments. For example, in March 2014, the PARADIGM-HF (Prospective Comparison of ARNI with ACEI to Determine Impact on Global Mortality and Morbidity in Heart Failure) trial of sacubitril/valsartan (Entresto, Novartis, Basel, Switzerland) was stopped early when investigators reported that the combination significantly reduced the risks of death and of hospitalization for heart failure (25). In April, the sponsor notified the FDA, which subsequently granted fast-track designation (which provides rolling review of the new drug application, more frequent communication with FDA

![Temporal Trends in Cardiovascular, Cancer, and Central Nervous System Drugs Entering Clinical Trials, 1990 to 2012](image)
reviewers, and other actions to expedite development) and priority review (which requires the FDA to make a decision within 6 months, rather than the standard 10-month timeline). The first full approval was granted by the FDA in July 2015, with approvals by authorities in Canada and Europe in October and November 2015, respectively.

Advances in translational science may yield further breakthroughs, and policymakers seeking to promote innovation in cardiovascular drug development should prioritize new incentives for such research, particularly given the demonstrated potential of recent cardiovascular translational efforts. For example, the discovery of proprotein convertase subtilisin/kexin type 9 (PCSK9) (26), identification of mutations in PCSK9 that reduce LDL cholesterol (27-29), and the ensuing validation of PCSK9 as a therapeutic target for the treatment of hypercholesterolemia (30,31) represent a recent success in the efficient translation of academic research into novel therapeutics. After the initial discovery in 2003, the first human clinical trial of a monoclonal antibody to PCSK9 was started in 2009 (32). In 2015, recognizing the potential benefit of these drugs for high-risk patients, the FDA approved alirocumab and evolocumab before the large cardiovascular outcome trials were completed. Other inhibitors of PCSK9 are now under development, including a RNA interference drug (33,34) and a therapeutic vaccine (35).

Our results also indicate that the pivotal trial results are frequently not published for drugs in which development is discontinued. This paucity of information, particularly on development failures, is problematic for investigators, as understanding why a drug fails may yield insights for other drugs in the same class and potentially guide efforts to repurpose failed drugs for new indications. Such information could also be useful to patients, who otherwise may be exposed to futile treatments or to unnecessary harms (36,37). In addition, even “failed” trials can yield valuable insights on the pathophysiology of disease and the validity of experimental systems and surrogate endpoints (such as in the case of torcetrapib and the use of LDL
cholesterol as a surrogate for cardiovascular outcomes). To improve reporting rates, recently, the National Institutes of Health issued a proposed regulation to require that trials for unapproved drugs are registered and that results are deposited in a public repository. If enacted, this rule may further incentivize investigators to share, at a minimum, summary repository. If enacted, this rule may further incentivize investigators to share, at a minimum, summary repository.

**STUDY LIMITATIONS.** We used publicly available sources and commercial databases to identify the reasons for discontinuation, but additional factors may have played a role in these failures. However, we were able to identify the stated reasons for discontinuation of development for most (86%) of the drugs in our study cohort. In addition, although our study period was chosen to allow sufficient time for follow-up, it is possible that some of the discontinued drugs may be approved, and more trial results may be published in the future.

**CONCLUSIONS**

Over the past 2 decades, fewer investigational cardiovascular drugs have entered clinical trials across all stages of development, though recently, more therapies have targeted novel biological pathways and have been sponsored by small and medium-sized companies. Most cardiovascular drugs fail in Phase 3 clinical trials due to inadequate efficacy or safety concerns, but cardiovascular drugs do not appear to be more likely to fail than drugs for other diseases. Given the increasing burden of cardiovascular disease globally, the declining pipeline of new therapies is concerning. Policymakers should focus their efforts on supporting research aimed at improving gaps in the understanding of the pathophysiological bases for cardiovascular disorders, as well as facilitating translational efforts to develop new cardiovascular therapeutics.

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KEY WORDS cardiovascular drug development, regulatory science, translational research

APPENDIX For supplemental figures, please see the supplemental appendix of this article.
Unilateral Carotid Body Resection in Resistant Hypertension
A Safety and Feasibility Trial

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HIGHLIGHTS

- First prospective feasibility and safety clinical trial on unilateral CB resection for the treatment of high blood pressure.
- In drug-resistant patients with hypertension, unilateral CB resection was feasible and safe.
- Unilateral CB resection lowered blood pressure by 26 mm Hg in 57% of patients with drug-resistant hypertension associated with a reduction in muscle sympathetic nerve activity and its baroreceptor reflex control.
- Whole drug equivalents were reduced in the responding patients.
- Responding patients had characteristics distinct to those that did not respond; these should allow patient selection for future CB modulation trials.
Summary

Animal and human data indicate pathological afferent signaling emanating from the carotid body that drives sympathetically mediated elevations in blood pressure in conditions of hypertension. This first-in-man, proof-of-principle study tested the safety and feasibility of unilateral carotid body resection in 15 patients with drug-resistant hypertension. The procedure proved to be safe and feasible. Overall, no change in blood pressure was found. However, 8 patients showed significant reductions in ambulatory blood pressure coinciding with decreases in sympathetic activity. The carotid body may be a novel target for treating an identifiable subpopulation of humans with hypertension. (J Am Coll Cardiol Basic Trans Science 2016;1:313–24) © 2016 The Authors. Published by Elsevier on behalf of the American College of Cardiology Foundation. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

Found bilaterally at the bifurcation of the common carotid artery, the carotid bodies (CBs) are strategically located to ensure that adequate oxygen is supplied to the brain. With the highest blood flow per tissue weight of any organ (1), they are exquisitely sensitive to small alterations in blood oxygen, carbon dioxide, pH, and blood flow itself (2,3). Despite their small size, the CBs exert powerful reflex effects on the respiratory and cardiovascular system (4) that have been preserved through evolution and are deemed pivotal for survival (5), perhaps due to their defensive reflex role. This powerful afferent system normally remains quiescent at sea level in resting conditions, but during hypoxia, the CBs are activated, increasing ventilation, increasing sympathetic activity, inducing alkalosis, and contributing to periodic breathing during sleep (6,7).

In patients with hypertension, the CBs exhibit both hyper-reflexia and aberrant discharge; in sleep apnea, the activation of the CBs is, in part, responsible for the excessive sympathetic activity and hypertension associated with this condition (8). Moreover, the hypertension evoked in a rat model of sleep apnea (by chronic intermittent hypoxia), is reliant on afferent activity generated by the CBs (9), and patients with hypertension may have exaggerated peripheral chemoreflex sensitivity to hypoxia (10,11). Additionally, acute reversible inactivation of the CBs, with hypoxia, caused a transient reduction in blood pressure (BP) and muscle sympathetic nerve activity (MSNA) in patients with hypertension (12). In rats with hypertension, severing the connection between the CBs and brain lowered both arterial pressure and sympathetic activity chronically (13). These data point toward the CBs as a therapeutic target to treat sympathetically mediated diseases (14). The global clinical problem and financial burden of hypertension continues to escalate (15), and 8% to 14% of the 1 billion patients with hypertension worldwide are drug resistant or intolerant (16). Therefore, new approaches for treating drug-resistant hypertension are justified, as are studies to identify the targets/mechanisms driving hypertension. We describe the first prospective proof-of-concept, safety and feasibility study of unilateral (u) CB excision from a cohort of patients with drug-resistant hypertension. We report, secondarily, on the proportion of these patients that showed a response in BP, the hypoxic ventilatory response (HVR), and MSNA.

Methods

Study Design and Patients. We present pooled results from 2 independent centers in which the primary endpoints were safety and feasibility of uCB excision in patients with drug-resistant hypertension. Secondary endpoints were to assess the proportion of

Dr. Lobo is supported by the Barts Hospital Charity; has served as a consultant to St. Jude Medical and ROX Medical; has received speaker fees from Cardiosonic; and has received an educational grant from Medtronic. Dr. Sobotka has served as a consultant to Cibiem, Inc.; has served as Chief Medical Officer, ROX Inc., and received salary and benefits; and has Stock Options in ROX Inc. and Cibiem. Dr. Leiter has served as a paid consultant to MAXIS Medical, Inc. Dr. Engelman is an employee of Cibiem, from which he receives a salary and has been awarded stock options. Dr. Nightingale’s institution has received financial support from Cibiem to carry out research in hypertension. Dr. Paton has served as a consultant for Cibiem. Drs. Narkiewicz and Ratcliffe contributed equally to this work.

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patients showing reductions in ambulatory blood pressure (ABP) and MSNA. Inclusion criteria were: age between 18 to 75 years; taking ≥3 antihypertensive medications, including a diuretic agent, at maximal tolerated dose; no evidence of causes of secondary hypertension following thorough biochemical, clinical, and imaging assessment (detailed in Supplemental Table 1); office systolic blood pressures (OSBP) ≥150 mm Hg; daytime mean ABP ≥135 mm Hg (Table 1, Supplemental Table 2).

There was no control group because this was a first-in-man, safety and feasibility study. Exclusion criteria are detailed in Supplemental Table 3. A total of 113 patients were recruited from specialist hypertension clinics at Bristol, United Kingdom; Gdansk, Poland; and London, United Kingdom. Following screening in Bristol and Gdansk, 15 patients (7 men and 8 women) were eligible, including 3 participants who had undergone renal denervation and 2 with sleep apnea who were on continuous positive airway pressure. All patients gave written informed consent. The study complied with the Declaration of Helsinki, was approved by the Central Bristol research ethics committee (12/SW/0277) and the Medical University of Gdansk Independent Bioethics Commission for Research (NKBBN/398/2012), and was registered at ClinicalTrials.gov (NCT01745172 and NCT01729988).

Informed consent was obtained from all patients.

STUDY PROTOCOL. Screening. Screening occurred 44 ± 15 days before the baseline visit. Clinical history, examination, and blood tests were taken to ensure that participants met entry criteria (see the previous text, and Supplemental Table 3). Office blood pressure (OBP) and 24-h ambulatory BP monitoring were recorded; for most patients, the latter commenced after observing antihypertensive pill administration (n = 11). A polysomnography study was performed. Patients were asked to keep a home blood pressure (HBP) and a medication diary for at least 10 days; this was the established practice and was a surrogate marker for drug compliance at the time this study commenced. Computed tomographic angiography of the carotid arteries was performed to define the carotid anatomy, and to assess the extent of atheromatous plaque/calcification, if any, and location of carotid body as described previously (17). Participants with high bifurcations, significant atheroma, unidentifiable carotid bodies, or carotid bodies inaccessible via the lateral surgical approach (see the following text) were excluded.

Study sessions. Visits were at baseline (27 ± 11 days pre-operative) and at 1, 3, 6, and 12 months post-CB resection. Following this clinical review, a blood sample (including a full blood count) (Tables 1 and 2) was taken and OBP, 24h ABP (at 3-, 6-, and 12-month follow-ups), beat-to-beat BP, MSNA, baroreflex sensitivity (BRS), and HVR were measured. Polysomnography was repeated 1 and 3 months after uCB resection, and HBP and medication diaries were repeated at 3 months post-operatively.

PROCEDURE. Safety monitoring.

1. Adverse event reporting. Adverse events were reported as per local sponsor guidelines, in line with ICH Good Clinical Practice recommendations, and were collated by the independent Clinical Research Organisation monitoring the studies and reviewed by the Clinical Events Committee formed by Cibiem.

2. HVR. The HVR was measured before and after uCB to assess any effect on chemoreflex sensitivity. Using an established poikilocapnic hypoxic protocol (18), patients were switched from breathing room air to 100% N₂ for up to 10 to 30 s. The procedure was repeated 5 to 10 times to achieve a range of SpO₂ from 100% to 75% while measuring respiratory frequency and tidal volume using spirometry (AD Instruments, Sydney, Australia). The HVR was calculated by assessing the largest 3 subsequent breaths during/following inhalation of 100% N₂. Minute ventilation was plotted against the SpO₂, and the slope of the regression reflects the HVR.

3. Sleep studies. Sleep data were acquired using Embla Sleep Systems (Embla Systems Inc., Thornton, Colorado) and analyzed using REMlogic software (Embla Systems Inc.). Post-uCB resection, sleep studies were performed at 1 and 3 months for 11 patients and at 1 month only for the remaining patients. The sleep studies were scored by an independent single scoring specialist center using international guidelines from the American Academy of Sleep Medicine (19).

4. Medications. Patients’ antihypertensive medicines were maintained at baseline values to minimize the effect of medication changes on BP after uCB unless there were strong clinical grounds for the management of symptomatic hypotension or other adverse events.

BP monitoring. OBP was taken in the sitting position using an automatic oscillometric monitor (Omron 705IT, Omron Healthcare Europe, Hoofddorp, the Netherlands). BP was measured according to protocol-specified guidelines on the basis of the European Society of Hypertension recommendations (20) and National Institute for Health and Care Excellence guidelines. The 24-h ABP (Spacelabs Healthcare, Snoqualmie, Washington) data was acquired at least
TABLE 1 Demographics, Screening Visit, Baseline, Number of Follow-Up Medications, Hemodynamic, and Respiratory Data

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<th></th>
<th>Screening</th>
<th>Baseline</th>
<th>1 Month</th>
<th>3 Months</th>
<th>6 Months</th>
<th>12 Months</th>
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<td>Age, yrs</td>
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<td></td>
<td></td>
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<tr>
<td>Height, m</td>
<td>1.69 ± 0.02</td>
<td></td>
<td></td>
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<td>Weight, kg</td>
<td>88.5 ± 4.1</td>
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<td></td>
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</tr>
<tr>
<td>BMI, kg/m²</td>
<td>31.0 ± 1.2</td>
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<tr>
<td>Number of drugs</td>
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<td>5.7 ± 0.6</td>
<td>5.1 ± 0.5</td>
<td>5.1 ± 0.4</td>
<td>4.9 ± 0.4</td>
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<td>Office</td>
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<tr>
<td>SBP, mm Hg</td>
<td>180 ± 6</td>
<td>168 ± 7</td>
<td>146 ± 8</td>
<td>153 ± 9</td>
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<td>114 ± 6</td>
<td>117 ± 5</td>
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<tr>
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<td>69 ± 3</td>
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<td>DBP, mm Hg</td>
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<td>97 ± 6</td>
<td>100 ± 6</td>
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<tr>
<td>MAP, mm Hg</td>
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<tr>
<td>PP, mm Hg</td>
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<td>64 ± 4</td>
<td>63 ± 3</td>
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</tr>
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<td>Ambulatory night</td>
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</tr>
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<td>SBP, mm Hg</td>
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<td>PP, mm Hg</td>
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<tr>
<td>Ambulatory overall</td>
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<td>SBP, mm Hg</td>
<td>163 ± 4</td>
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<td>97 ± 4</td>
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<tr>
<td>MSNA incidence, per 100 heart beats</td>
<td>77.2 ± 4.0</td>
<td></td>
<td>75.7 ± 3.9</td>
<td>73.1 ± 4.2</td>
<td>72.4 ± 3.5</td>
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<tr>
<td>MSNA frequency, per min</td>
<td>–</td>
<td>50.8 ± 2.1</td>
<td>49.4 ± 3.1</td>
<td>49.1 ± 1.8</td>
<td>46.3 ± 2.6</td>
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<tr>
<td>BRS, % x/mm Hg</td>
<td>–</td>
<td>-1.16 ± 0.26</td>
<td>-1.21 ± 0.2</td>
<td>-1.73 ± 0.19</td>
<td>-1.54 ± 0.26</td>
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<tr>
<td>HRV, LF-HF</td>
<td>–</td>
<td>2.1 ± 0.5</td>
<td>2.2 ± 0.6</td>
<td>1.4 ± 0.3</td>
<td>2.0 ± 0.4</td>
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<tr>
<td>HVR, l/min=SpO2</td>
<td>–</td>
<td>-0.44 ± 0.04</td>
<td>-0.43 ± 0.07</td>
<td>-0.39 ± 0.11</td>
<td>-0.56 ± 0.13</td>
<td>-0.41 ± 0.06</td>
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<td>Respiratory rate, per min</td>
<td>–</td>
<td>15.5 ± 1.4</td>
<td>15.2 ± 1.0</td>
<td>16.6 ± 1.4</td>
<td>16.4 ± 0.9</td>
<td></td>
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<tr>
<td>Tidal volume, l</td>
<td>–</td>
<td>0.63 ± 0.06</td>
<td>0.63 ± 0.09</td>
<td>0.59 ± 0.04</td>
<td>0.58 ± 0.05</td>
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<tr>
<td>Minute ventilation, l/min</td>
<td>–</td>
<td>8.9 ± 0.8</td>
<td>8.5 ± 0.6</td>
<td>9.3 ± 0.7</td>
<td>9.1 ± 0.7</td>
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<tr>
<td>Hb, g/dl</td>
<td>–</td>
<td>14.2 ± 0.3</td>
<td>14.1 ± 0.3</td>
<td>14.1 ± 0.4</td>
<td>14.3 ± 0.3</td>
<td>14.2 ± 0.4</td>
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<tr>
<td>HbA1C (DCCT), %</td>
<td>–</td>
<td>5.9 ± 0.2</td>
<td>5.8 ± 0.2</td>
<td>6.0 ± 0.2</td>
<td>5.8 ± 0.2</td>
<td>5.7 ± 0.2</td>
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</table>

Values are n/n (N) or mean ± SEM.

BMI = body mass index; BRS = baroreceptor reflex sensitivity of muscle sympathetic nerve activity; DBP = diastolic blood pressure; DCCT = Diabetes Control and Complications Trial; Hb = hemoglobin; HbA1C = glycated hemoglobin; HF = high frequency; HR = heart rate; HRV = heart rate variability; HVR = hypoxic ventilatory response; LF = low frequency; MAP = mean arterial pressure; MSNA = muscle sympathetic nerve activity; PP = pulse pressure; SBP = systolic blood pressure; TV = tidal volume.

Once hourly during sleep and twice hourly during the day (night: 12:00 AM to 5:59 AM; day: 6:00 AM to 11:59 PM). The HBP (Omrón 705IT) was averaged across the last 2 of 3 morning and 3 evening readings daily for 10 to 14 days; a medication diary was kept with HBP readings.

Microneurography and hemodynamic recordings. During each study visit, continuous BP recordings were measured using a Finometer (Finapres Medical Systems, Amsterdam-Zuidoost, the Netherlands), which was calibrated to the BP measured in the same arm using an automated cuff, and a 3-lead electrocardiograph was used for continuous monitoring of heart rate; these recordings were used to calculate BRS. Multiunit MSNA was recorded by microneurography in the peroneal nerve at the fibular head (21) using tungsten microelectrodes (FHC Inc., Bowdoin, Maine). Heart rate, BP, and MSNA were measured and recorded continuously using a data acquisition program (LabChart, AD Instruments). Following insertion of the microelectrode, resting hemodynamics and MSNA recordings were collected.
over 5 to 20 min of quiet supine rest. Bursts were identified, and their frequency (Hz) and incidence (per 100 heart beats) were measured. Heart rate variability (HRV) was calculated using an add-on in LabChart (AD Instruments) using spectral analysis conforming to previous guidelines (22), with high from 0.15 to 0.4 Hz, low frequency from 0.04 to 0.15 Hz, and very low frequency from 0.0033 to 0.04 Hz.

**BRS measurement on the basis of MSNA burst area.**
Offline, MSNA was normalized to the amplitude of the largest burst in the recording and was represented as a percentage of this burst. For each cardiac cycle, the associated diastolic blood pressure (DBP) was determined and collected into 1-mm Hg bins. The peak, beginning, and end of every MSNA burst were marked in data acquisition software (Spike2, Cambridge Electronic Design, Cambridge, United Kingdom). The area of the burst was calculated as the integral of MSNA between the beginning and the end of the burst (units of % · s). Each cardiac cycle was associated with the accompanying MSNA burst area (MSNA burst area = 0 AU if no burst occurred in that cycle). The lowest DBP in each cardiac cycle over the entire range of DBPs obtained in each subject. The spontaneous MSNA BRS was calculated using DBP and a similar calculation to that described previously (23).

**SURGERY.** The surgical removal of the CB was performed following the procedure described by Winter (24). Under either general or local anesthesia with sedation, an incision was made over the anterior aspect of the sternocleidomastoid muscle, one-third of the distance between the angle of the mandible and the clavicle, and over the region of the carotid bifurcation as identified via ultrasound/computed tomography angiography. The sternocleidomastoid muscle was retracted laterally along with the internal jugular vein to expose the carotid bifurcation. By gentle retraction of the external carotid artery (in some cases, the superior thyroid artery was cut to enhance retraction), the intercarotid septum was exposed. Tissue within this septum was isolated from the internal and external carotid arteries, and a ligature was placed at the saddle of the bifurcation. The septal tissue was excised as close to the ligature as possible. The surgeries in some cases involved ligation of the pedicle, and in others cautery over the saddle.

**HISTOLOGY.** Resected tissue was fixed with 10% formalin, embedded in paraffin, sectioned (50- to 100-μm thick), and stained with hematoxylin and eosin. The presence of glomus cells and organ margins were checked and photomicrographs were taken.

**ANALYTICAL METHODS AND STATISTICAL ANALYSIS.**
All analyses were conducted blind with respect to the sequence of visits and whether the patient was a BP responder or nonresponder (see the following text).
All measurements of MSNA burst area and BRS were calculated by importing acquired data into MATLAB 8.0 (2012b, The MathWorks, Natick, Massachusetts), and statistical tests of these measures were

| TABLE 2 Demographics and Screening Visit Data for Responders and Nonresponders |
|-------------------------------|-------------------------------|-----------------|-----------------|
|                              | Responders                  | Nonresponders   | p Value         |
| Male/female                   | 3/3                          | 4/2             |                 |
| Age, yrs                      | 55 ± 2                      | 52 ± 3          | 0.34            |
| Height, m                     | 1.68 ± 0.03                 | 1.72 ± 0.03     | 0.40            |
| Weight, kg                    | 89.2 ± 5.8                  | 91.1 ± 6.4      | 0.83            |
| BMI, kg/m²                    | 31.6 ± 1.8                  | 30.9 ± 1.6      | 0.78            |
| Antihypertensive drugs        | 5.8 ± 0.5                   | 5.7 ± 0.6       | 0.94            |
| Office screening              |                              |                 |                 |
| SBP, mm Hg                    | 187 ± 11                    | 170 ± 7         | 0.68            |
| DBP, mm Hg                    | 94 ± 6                      | 107 ± 9         | 0.26            |
| MAP, mm Hg                    | 118 ± 8                     | 128 ± 8         | 0.36            |
| PP, mm Hg                     | 70 ± 8                      | 64 ± 4          | 0.54            |
| HR, beats/min                 | 75 ± 6                      | 72 ± 6          | 0.75            |
| Ambulatory screening day      |                              |                 |                 |
| SBP, mm Hg                    | 171 ± 8                     | 162 ± 5         | 0.39            |
| DBP, mm Hg                    | 101 ± 7                     | 98 ± 7          | 0.27            |
| MAP, mm Hg                    | 124 ± 7                     | 119 ± 6         | 0.57            |
| PP, mm Hg                     | 71 ± 4                      | 64 ± 4          | 0.30            |
| Ambulatory screening night    |                              |                 |                 |
| SBP, mm Hg                    | 148 ± 7                     | 143 ± 2         | 0.56            |
| DBP, mm Hg                    | 83 ± 5                      | 84 ± 4          | 0.85            |
| MAP, mm Hg                    | 105 ± 5                     | 104 ± 3         | 0.90            |
| PP, mm Hg                     | 65 ± 6                      | 59 ± 4          | 0.44            |
| Ambulatory screening overall  |                              |                 |                 |
| SBP, mm Hg                    | 167 ± 7                     | 158 ± 3         | 0.32            |
| DBP, mm Hg                    | 97 ± 6                      | 95 ± 6          | 0.84            |
| MAP, mm Hg                    | 120 ± 6                     | 116 ± 5         | 0.71            |
| PP, mm Hg                     | 70 ± 4                      | 63 ± 4          | 0.28            |
| MSNA incidence, per 100 heart beats | 82.5 ± 4.9                  | 74.1 ± 6.4      | 0.31            |
| MSNA frequency, per min       | 51.7 ± 2.9                  | 47.7 ± 4.7      | 0.46            |
| BRS, % s/mm Hg                | -1.23 ± 0.24                | -1.15 ± 0.59    | 0.89            |
| HRV, LF/HF                    | 2.5 ± 0.8                   | 2.0 ± 0.7       | 0.66            |
| HVR, l/min/SpO2               | -0.50 ± 0.05                | -0.32 ± 0.06    | 0.027           |
| Respiratory rate, per min     | 18.2 ± 2.0                  | 11.8 ± 1.1      | 0.025           |
| Tidal volume, l               | 0.50 ± 0.05                 | 0.84 ± 0.09     | 0.003           |
| Minute ventilation, l/min     | 8.9 ± 1.2                   | 9.2 ± 1.2       | 0.87            |
| HbA1c, (% DCCT)               | 5.99 ± 0.27                 | 5.70 ± 0.16     | 0.41            |
| Hb, g/dl                      | 14.40 ± 0.43                | 14.48 ± 0.29    | 0.89            |

Values are n or mean ± SEM. All variables passed normality except day ambulatory SBP. The median (first quartile, third quartile) were ambulatory systolic blood pressure (p = 0.834): responders = 169.5 (156.2, 173.7) and nonresponders = 162.3 (152.9, 172.5). Abbreviations as in Table 1.
Patients maintained their ventilatory response to hypoxia as the average baseline HVR (−0.4 ± 0.11/min/% SpO2) (Table 1) was not changed after uCB resection at any time point. There were no changes in breathing patterns during sleep after uCB (Supplemental Table 5), and although blood oxygen fell to lower minimal levels during desaturation episodes (from 87 ± 1% to 81 ± 1%; p < 0.05), there were no changes in the apnea-hypopnea index, apnea duration, baseline blood oxygen saturation, and average blood desaturation (Supplemental Table 5).

FEASIBILITY. On the basis of its visualization on computed tomography scans (Figure 1), a CB was resected from either the right (n = 11) or left side (n = 4). Characteristic glomus tissue was found subsequently in histological sections of the resected specimen in all (Figure 1) but 1 patient; in this patient we observed no adverse effects, changes in BP, or changes in any of the other measured variables at all follow-up visits.

BP, MEDICATION, AND AUTONOMIC INDEXES IN ALL PATIENTS (N=15). There was no significant change in either ASBP or OSBP at any time following uCB resection compared with the corresponding values at screening and baseline (Figure 2, Table 1) (n = 15); a similar finding was observed for HBP (Supplemental Figure 1). Notably, at all follow-up examinations there was no statistically significant difference between screening and baseline OSBP or 24-h ASBP (Table 1), indicating stability of BP in the run-up to uCB resection. Also, there were no changes in MSNA (frequency or incidence), BRS, heart rate variability, HVR and minute ventilation (Table 1), or medication between screening and baseline (Supplemental Table 2). We next determined if there was a proportion of patients that showed a reduction in BP and whether this correlated with any other measured variable.

PROPORTIONATING AND CHARACTERIZING RESPONDING PATIENTS. We defined a responder as a participant with evidence of glomus cells in the resected tissue (as determined histologically) and a ≥10-mm Hg drop in ABP at the 3-month follow-up visit to allow time for patient recovery and the resolution of adverse events. A nonresponder was defined as having evidence of glomus cells in the resected tissue, but did not show a ≥10-mm Hg fall in ambulatory BP at 3-month follow-up. On the basis of these definitions, there were 8 responders (i.e., 53.3%; 95% confidence interval: 26.6% to 78.7%) who showed significant reductions in ASBP at 3 months and 6 nonresponders (Figure 3); glomus cells were not found in 1 patient (see the previous text). No patient

SAFETY. There were 2 serious adverse events consisting of prolonged hospitalization of patients with BP that was difficult to control. One of the events occurred shortly after the CB removal procedure, and this event was judged by the Clinical Events Committee (CEC) to be “possibly related” to the unilateral removal of the CB. In the other patient, multiple hospitalizations occurred for BP control before and after uCB removal, and the hospitalizations bore no consistent temporal relationship to the CB removal. These hospitalizations were, therefore, judged by the CEC to be “unrelated” or “unlikely to be related” to the uCB.

In 1 patient with pre-existing moderate obstructive sleep apnea (OSA), sleep-disordered breathing (SDB) worsened after uCB. This was not noted as an adverse event by the study site. The CEC felt, however, that given that SDB was a pre-existing disease in this patient and the apnea-hypopnea index increased from 20 events/h at baseline to 74 events/h 3 months post-carotid body removal, worsening SDB should be noted as an adverse event. The patient was treated with continuous positive airway pressure, and the apnea-hypopnea index decreased substantially. The adverse events and polysomnography are shown in Supplemental Tables 4 and 5, respectively.

cWASCONducted in Prism version 2.0 (GraphPad Software, Inc., La Jolla, California). Results are presented as mean ± SEM. A 2-sided probability value of p < 0.05 was considered statistically significant. Statistical comparisons of groups were assessed by 1-way analysis of variance (ANOVA) for parametric data or Kruskal-Wallis ANOVA on ranks for data that were not normally distributed. A 2-way ANOVA with Sidak (between groups) and Tukey (within groups) post hoc tests was conducted to compare data across and within groups at different follow-ups. Serial within-group comparisons were subjected to repeated measures ANOVA. All variables analyzed with ANOVA passed the D’Agostino & Pearson normality test and also Bartlett’s test for equal variance. All variables passed normality except for day ambulatory systolic blood pressure (ASBP), which was analyzed with Kruskal-Wallis test and expressed as median (first quartile, third quartile). Medications were calculated as a percentage of the maximal recommended dose for hypertension. For each patient, the whole drug as a percentage of the maximal recommended dose for quartile, third quartile). Medications were calculated within-group comparisons were subjected to repeated measures ANOVA. All variables analyzed with ANOVA passed the D’Agostino & Pearson normality test and also Bartlett’s test for equal variance. All variables passed normality except for day ambulatory systolic blood pressure (ASBP), which was analyzed with Kruskal-Wallis test and expressed as median (first quartile, third quartile). Medications were calculated as a percentage of the maximal recommended dose for hypertension. For each patient, the whole drug equivalents (sum of the percentage of the maximal recommended dose) were calculated on the basis of doses quoted in the British National Formulary.

RESULTS

SAFETY. There were 2 serious adverse events consisting of prolonged hospitalization of patients with BP that was difficult to control. One of the events occurred shortly after the CB removal procedure, and this event was judged by the Clinical Events Committee (CEC) to be “possibly related” to the unilateral removal of the CB. In the other patient, multiple hospitalizations occurred for BP control before and after uCB removal, and the hospitalizations were considered statistically significant. Statistical comparisons of groups were assessed by 1-way analysis of variance (ANOVA) for parametric data or Kruskal-Wallis ANOVA on ranks for data that were not normally distributed. A 2-way ANOVA with Sidak (between groups) and Tukey (within groups) post hoc tests was conducted to compare data across and within groups at different follow-ups. Serial within-group comparisons were subjected to repeated measures ANOVA. All variables analyzed with ANOVA passed the D’Agostino & Pearson normality test and also Bartlett’s test for equal variance. All variables passed normality except for day ambulatory systolic blood pressure (ASBP), which was analyzed with Kruskal-Wallis test and expressed as median (first quartile, third quartile). Medications were calculated as a percentage of the maximal recommended dose for hypertension. For each patient, the whole drug equivalents (sum of the percentage of the maximal recommended dose) were calculated on the basis of doses quoted in the British National Formulary.
with a left CB resected and/or prior renal nerve denervation responded. In the 8 responders, day ASBP decreased at the 3-month (−23 ± 3 mm Hg; p = 0.0005), 6-month (−26 ± 4 mm Hg; p = 0.0021), but not 12-month (−12 ± 8 mm Hg; p = 0.22) follow-up compared with screening (Figure 3A). Night-time ASBP was also reduced at 3-month (−20 ± 4 mm Hg; p < 0.0243), 6-month (−16 ± 5 mm Hg; p < 0.047) and at 12-month follow-ups (−15 ± 6 mm Hg; although p = 0.067) compared with screening (Figure 3B) with 24-h ASBP following a similar time-course (Figure 3C). Comparing both day and night ASBP between responders and nonresponders, there were significant differences between patient groups at all time points (Figures 3A and 3B) (p < 0.05 to p < 0.01). Regarding OSBP, responding patients exhibited a reduction at 1 month (−52 ± 8 mm Hg; p = 0.006), 3 months (−46 ± 8 mm Hg; p < 0.005), and 6 months (−35 ± 7 mm Hg; p < 0.0088), but not at 12 months compared with both screening and baseline (Figure 3D). There was no change in the variability of ASBP. For ASBP and OSBP, there were no sex-related differences (Fisher exact test). The HBP was also reduced in responders but not nonresponders at 3 months (Supplemental Figure 1).

ANTIHYPERTENSIVE MEDICATIONS. Among responders, across all study time points, there was a significant difference in whole-dose equivalents (WDE) (p = 0.0009; 4.5 ± 0.6 WDE at screening and baseline, falling to 3.5 ± 0.6 WDE by 6 and 12 months). There was also a trend toward reductions in both the number of medications (p = 0.06) and medication classes (p = 0.06). The were no changes in WDE (0.98), the number of medications (p = 0.15), or medication classes (p = 0.15), among nonresponders.

MSNA FOLLOWING CB RESECTION. Neither baseline MSNA burst frequency nor incidence differed between responders and nonresponders (Table 2, Figures 4A and 4B) (p = 0.31 and p = 0.46, respectively). However, compared with baseline, responders exhibited a decrease in total MSNA burst area/min at 3 months (−374 ± 102%-s/min; p = 0.0137) and 6 months (−520 ± 125%-s/min; p = 0.0296), but not at the 12-month follow-up (p = 0.74) (Figure 4C). Furthermore, total MSNA burst area/min in the responders was lower than in nonresponders at 3-month (−374 ± 108%-s/min vs. 281 ± 174%-s/min; p < 0.0154), 6-month (−165 ± 135%-s/min vs. 16 ± 193%-s/min; p < 0.025) and at 12-month follow-up (−166 ± 38%-s/min vs. 386 ± 123%-s/min; p = 0.06) (Figure 4C). In contrast, total MSNA burst area/min compared with baseline did not change at any follow-up time in the nonresponders (Figure 4C).

BAROREFLEX MODULATION OF MSNA. In responders, BRS improved as revealed by a decrease at 6-month (−1.50 ± 0.18%-s/mm Hg; p < 0.0245), but not at 12-month follow-up (−0.96 ± 0.63%-s/mm Hg;
p = 0.52) compared with baseline (Figure 4D). There was no change in BRS relative to baseline in non-responders at any time point (Figure 4D) (p > 0.05).

CHARACTERISTIC DISTINCTIONS BETWEEN RESPONDERS AND NONRESPONDERS. Before surgery and compared with nonresponders, responders had a higher hypoxic ventilatory response (p < 0.027) and faster ventilatory frequency (p < 0.025) (Table 2) at baseline consistent with higher peripheral chemoreflex sensitivity and drive, respectively. Moreover, they consistently had the right carotid body removed.

DISCUSSION

The present first-in-man, proof-of-principle study was concerned with investigating the safety and feasibility of unilateral surgical resection of CB as a therapy in patients with drug-resistant hypertension. We found that this procedure was safe as evidenced by an absence of persistent serious adverse events, maintenance of a ventilatory response to hypoxia, and with 1 exception, no major alteration in SDB. With the finding of glomus cells in resected tissue from 14 of 15 patients in our study, the feasibility of the surgical approach has been demonstrated.

As recently reviewed, CB resection (unilateral and bilateral) has been performed historically for the treatment of dyspnea in patients with asthma and chronic obstructive pulmonary disease (14). In 1 of these studies, a chronic reduction in BP was noted in patients who had hypertension, which was maintained for 6 months when the study ended; there was no BP change in the normotensive group (25).
Additionally, a retrospective study showed BP reduction in patients with hypertension following uCB tumor resection (26). Although we saw a worsening of SDB in 1 patient with pre-existing OSA, a systematic analysis of sleep studies in the other patients in this study and in other studies in which the CB was denervated/removed (27) has not demonstrated any consistent change in the severity of OSA. The causes of OSA are likely to be multifactorial, and for a given patient, the functional role of the CB in inducing or preventing OSA episodes may depend on the precise etiology of the apnea.

**CB DYSFUNCTION PREVALENCE IN HYPERTENSION.** Across all patients, there were no changes in ABP or OBP after uCB resection. This was not surprising given that hypertension has a heterogeneous etiology and chemoreflex hyper-reflexia was reported in only a subset of patients with cardiovascular disease (18). Hence, we performed a post hoc analysis using a BP reduction of >10 mm Hg in daytime ASBP at 3 months post-uCB resection to identify if a proportion of participants had responded, and if so, whether they exhibited any distinct physiological features. We found a substantial reduction in daytime ASBP in 8 patients (>20 mm Hg) that persisted for at least 6 months. The BP response after uCB resection wanes at 12 months, which may reflect compensation including that from the other CB. Notably, the level of BP in responders remained significantly lower relative to nonresponders at 12 months. It is encouraging that in these responding patients, medications were reduced compared with baseline, which may have blunted the magnitude of the BP response. Longer-term follow-up data is now crucial to determine the persistence of the BP-lowering effect following uCB resection. Nevertheless, the reduction in both ABP and medication suggests that CB modulation therapy may go beyond a pharmacological treatment for a subset of patients.

**MECHANISM OF ACTION OF CB RESECTION.** In the responders (but not the nonresponders), MSNA total activity was reduced over a similar time course to the ABP, indicating reduced vasomotor tone; this is consistent with data from hypertensive rats (13) and data seen transiently using hyperoxia to inhibit CB afferent activity in humans with hypertension (12). We also noted an improvement in MSNA BRS in the responder but not in the nonresponder patients, which could contribute mechanistically to the lowering of BP. This finding is consistent with the known antagonism between peripheral chemoreceptor and baroreflex function (10).
The elevated HVR and higher respiratory frequency in the responders versus nonresponders is supportive of aberrant hyper-reflexia and high CB drive, respectively. The elevated HVR is consistent with peripheral chemoreceptor hyper-reflexia in hypertension, as reported in animals (13) and humans with hypertension (8,11). It may be possible to use HVR and respiratory frequency (and hyperoxia to depress the CB) (12) to pre-select the patients with hypertension who are most likely to benefit from CB modulation therapy, assuming that their arteries and arterioles are able to vasorelax.

**STUDY LIMITATIONS.** As a first-in-man, safety and feasibility study, we did not include a control group. However, in the 1 patient in whom we found no evidence of glomus cells in the resected tissue, we failed to see a fall in BP. Notwithstanding the recruitment difficulties associated with a surgical intervention, we acknowledge that the absence of a control arm and the low number of patients are limitations of the present study. A percutaneous catheter-based approach to ablate the CB selectively may be better tolerated, for example.

Although patients were fully assessed in specialist hypertension clinics before study entry, drug non-adherence cannot be ruled out. Notably, we fully adhered to the established drug adherence procedures at the time the study was initiated (see the Methods sections). There were no statistical differences between screening and baseline OSBP, which may partly overcome concerns regarding a Hawthorne effect and regression to the mean.

**REASONS FOR DYSFUNCTIONAL CB.** The issue of why the CB develops hypersensitivity is unclear, but may include hypoperfusion as suggested in heart failure (2). In the hypertensive condition, this may be a result of stenosis caused by atheroma or arteriole hypertrophy; the latter may be induced by elevated sympathetic activity to the CB and/or angiotensin II. Inflammation is also present in the CB of hypertensive rats (28) and may trigger release of cytokines, chemokines, and reactive oxygen species that could...
also contribute to its hyperactive state. In rodents subjected to chronic intermittent hypoxia, a condition causing hypertension and reflected in humans with sleep apnea, there is a change in the balance of gasotransmitters including H\textsubscript{2}S and CO (29) and purinergic mechanisms (30); whether these occur in the CBs of human patients with hypertension is unknown.

Turning to the nonresponders, there are several explanations for lack of BP improvement. Anatomical variations in the distribution of the CB may result in insufficient glomus tissue being resected. We acknowledge the heterogeneous etiology of hypertension and would not expect a BP response in individuals with nondysfunctional CBs or mechanisms unrelated to CB dysfunction. Even in patients with CB dysfunction, it may be that the contralateral CB predominates in driving up BP. We do not rule out stiffened arteries that are unable to regain compliance even with a reduction in sympathetic tone.

CONCLUSIONS

This is the first study that places CB hyper-reflexia in context of a disease state in humans and demonstrates the potential benefit of modifying that activity.

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COMPETENCY IN MEDICAL KNOWLEDGE: CB modulation therapy could become a novel therapeutic strategy for treating hypertension in some individuals. It remains unclear whether there is a dominant (i.e., left or right) CB for BP control, but this could be established in future trials. Given the response rate indicated herein (8 of 15 patients), future studies need to devise protocols, which might include the HVR, to select those patients with aberrant CB activity, as these are predicted to respond to treatment.

TRANSLATIONAL OUTLOOK 1: Although surgical resection was feasible, less invasive procedures need to be devised such as percutaneous ablation catheter technology; such an approach is ongoing, where an ablation catheter is advanced into the jugular vein and, using intravascular ultrasound, positioned precisely at the level of the bifurcation of the common carotid artery to ablate the CB.

TRANSLATIONAL OUTLOOK 2: Reverse translation is needed to identify the molecular mechanisms that cause both the hyper-reflexia and tonicity of the CB in conditions of human hypertension; this would reveal further opportunities for CB modulation therapy.

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KEY WORDS afferent drive, baroreceptor reflex, hypertension, hypoxia, peripheral chemoreceptor, sympathetic nervous system

APPENDIX For a supplemental figure and tables, please see the online version of this article.
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