Influence of ivabradine on reverse remodelling during mechanical unloading

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Aims
Ivabradine (Iva) has shown beneficial structural and functional effects in clinical and experimental heart failure (HF), but its action in combination with mechanical unloading (MU), such as during treatment with left ventricular assist devices (LVAD), is unknown. The aim of this study was to investigate the effects of Iva during MU, in a rodent model of HF.

Methods and results
We studied the chronic effects (4 weeks) of Iva (10 mg/kg/day) alone and in combination with MU [induced by heterotopic abdominal heart transplantation (HATx)] on whole-heart and cellular structure, function, and excitation–contraction (E–C) coupling in a rodent (rat) model of HF, 12 weeks post-left coronary artery (LCA) ligation. Effects of Iva were compared with those of β-blockade using metoprolol [(Met), 250 mg/kg/day]. Iva, but not Met, reversed myocardial fibrosis, alone and in combination with MU. MU-induced restoration of deranged E–C coupling was enhanced by Iva to a greater extent than Met; both Iva and Met enhanced the recovery of the Ca2+ transient amplitude and the sarcoplasmic reticulum (SR) Ca2+ content, but Iva alone maintained MU-induced normalization of L-type Ca2+ current and t-tubule abnormalities. Met prevented MU-induced reduction in the myocardial size (myocardial atrophy); Iva had no effect on this parameter.

Conclusion
Iva shows beneficial structural and E–C coupling effects during MU: Iva reverses myocardial fibrosis and enhances the restoration of deranged E–C coupling, displaying more beneficial effects than that of Met. These results suggest that Iva may prove effective in enhancing functional recovery in heart failure patients receiving LVAD therapy.

Keywords
Heart rate • Heart failure • Cardiovascular surgery • Ventricular assist device • Reverse remodelling

1. Introduction
Left ventricular assist devices (LVADs) are increasingly used in end-stage heart failure (HF) treatment, primarily as bridge to transplantation.1 Mechanical unloading (MU) of the failing LV with LVADs is associated with reverse remodelling of diseased myocardium, and significant beneficial cellular, molecular, electrophysiological, and structural changes.2 Despite these effects, adequate functional recovery permitting device explantation [bridge to recovery (BTR)] remains rare (4–10%).2,3 The reasons for this disparity are unknown, but are likely due to detrimental changes induced by prolonged unloading,4,5 such as myocardial fibrosis,6 deranged excitation–contraction (E–C) coupling,7 and myocardial atrophy.5 Prevention of these effects by pharmacological agents has attracted considerable interest.8–11

Ivabradine (Iva) is a heart rate reduction (HRR) agent that selectively inhibits the pacemaker hyperpolarization-activated If current in the sinoatrial node (SAN), causing a dose-dependent HRR without effects on conduction, inotropy, or blood pressure.12 Iva has demonstrated added clinical benefit when combined with β-blockade in HF therapy,13 showing beneficial LV structural remodelling effects in HF,14 and cardiac transplant patients.15,16 In post-infarction rodent HF, Iva shows multiple beneficial effects: Iva improves whole-heart systolic17–20 and diastolic function,21 decreases interstitial18,19,21 and
peri-vascular fibrosis, increases angiogenesis, and myocardial perfusion, improves Ca$^{2+}$ handling, and decreases mortality and ventricular excitability. The effects of Iva during MU are unknown. Iva’s ability to improve cardiac fibrosis, Ca$^{2+}$ handling, and LV structure/size, three proposed key determinants of functional recovery during LVAD support, highlights an attractive potential use of Iva in enhancing myocardial recovery in patients receiving LVAD support. Therefore, in this study, we tested the hypothesis that Iva would positively enhance reverse remodelling, and promote E–C coupling recovery during MU. We tested this hypothesis by examining the chronic effects of Iva on whole-heart/cell size, function, fibrosis, E–C coupling, and t-tubule structure in a rodent model of HF, and during MU, and compared them to those of β-blockade with Met.

2. Methods
The investigation conformed to the Guide for the care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No.85–23, revised 1996). Experiments were approved by the Harefield Heart Science Centre ethics review panel. (For extended methods, please refer to Supplementary material online).

2.1 Animal models
2.1.1 Induction of heart failure
HF was induced via permanent left coronary artery (LCA) ligation as previously described. Animals were anaesthetized with isoflurane (5% induction, 1.5% maintenance) mixed with O$_2$ (2–3 L/min), intubated and mechanically ventilated via a rodent ventilator, and a left thoracotomy performed. The pericardium was opened and heart visualized, and a left thoracotomy performed. The pericardium was opened and heart visualized, and a left thoracotomy performed. Acute 24 h mortality was 25% in the LCA ligation group and 0% in the sham group. Male syngeneic Lewis rats (10 weeks old ~200 g, Harlan, UK) were used for all experiments to avoid the need for immunosuppression following subsequent heterotopic abdominal transplant (HATx) in the MU groups. One week after surgery LV dimensions and function [ejection fraction (EF)] were assessed by transthoracic echocardiography (TTE) (Acuson SequoiaTM 256; Acuson, USA), as previously described. saline administration was 4 weeks. At the end of the treatment period change in EF was 4 weeks.

2.1.2 Animal anaesthesia, analgesia, and euthanasia
The adequacy of anaesthesia during all procedures was monitored by the loss of reflexes, degree of muscle relaxation, and respiration rate. Respiration rate and body temperature were monitored throughout all procedures and body temperature maintained at 37°C, via heating mat. Recipients were then assigned to three treatment groups receiving water containing: (i) Iva (Servier, France) 10 mg/kg/day, (ii) Met (Sigma, England) 250 mg/kg/day, and (iii) no treatment (MUHF + Iva, MUHF + Met and MUHF, respectively). Treatment was commenced 12 weeks after LCA ligation, immediately after transplantation, and for the duration of 4 weeks.

The effects of Iva and Met on non-transplanted failing hearts were also studied for comparison. HF animals were randomly allocated to three treatment groups: (i) Iva 10 mg/kg/day in drinking water, (ii) Met 250 mg/kg/day in drinking water, and (iii) no treatment receiving normal drinking water (HF + Iva, HF + Met and HF, respectively). Treatment duration was 4 weeks. At the end of the treatment period change in EF was measured via TTE (Supplementary material online, Table S1).

Weekly in vivo assessment of HR in non-anaesthetized animals was performed using implantable ECG telemetry transmitters (CA-F40 Data Sciences International, Minneapolis, MN, USA). Telemetry devices were implanted subcutaneously (SC), under general anaesthesia using isoflurane (5% induction, 1% maintenance) mixed with O$_2$ (2–3 L/min).

2.1.3 Histological analysis of myocardial fibrosis
Interstitial myocardial fibrosis was quantified using a well-established technique. Longitudinal slices were obtained from viable LV free wall, processed and incubated with the collagen specific dye picrosirius red. Stained sections were viewed under bright field and using red fluorescence filter at >20 magnification on a Zeiss Axioscop microscope with a Nikon DMX1200 camera, and Image fibrosis analysis was carried out using NIS elements 3.0 software (Nikon, UK). The relative area of myocardial interstitial fibrosis was calculated as the area fraction of the visual field staining picrosirius red [collagen area fraction (CAF)]. All morphometric data were collected by the same observer in a blinded fashion. CAF was measured at the end of the treatment period, i.e. 16 weeks after LCA ligation, in all treatment groups. To quantify pre-treatment levels of myocardial fibrosis in failing animals, CAF was also specifically measured in a separate group of HF rats (n = 4), 12 weeks after LCA ligation.

2.1.4 Cardiomyocyte studies
LV cardiomyocytes were isolated by standard enzymatic digestion using collagenase type 2 (1 mg/mL, Worthington) and hyaluronidase (0.6 mg/mL, Sigma) for 8–10 min and used within 6 h.
2.1.4.1 Assessment of sarcomere shortening and Ca^{2+} handling
Simultaneous assessment of sarcomere shortening and Ca^{2+} handling in field stimulated cardiomyocytes was performed using an Ionoptix system (Ionoptix Corporation, USA) and the Ca^{2+} sensitive dye Indo-1 (Indo-1 AM, Molecular Probes). In addition, sarcoplasmic reticulum (SR) Ca^{2+} content, fractional SR Ca^{2+} release (ratio of stimulated Ca^{2+} transient over caffeine-induced Ca^{2+} transient), SR Ca^{2+} uptake, and sodium–calcium exchanger (NCX) contribution to Ca^{2+} extrusion were assessed using rapid caffeine application, as previously described.\(^23\)

2.1.4.2 Assessment of electrophysiological parameters
Cells were studied using a MultiClamp 700A (Axon Instruments) in whole-cell patch configuration. Current–voltage relationships for L-type Ca^{2+} current were studied and normalized to cell capacitance as previously described.\(^23\)

2.1.4.3 Assessment of cell volume, t-tubules, and Ca^{2+} sparks with confocal microscopy
Cell volume and t-tubule organization were studied using the membrane binding dye di-8-Anapts (Molecular Probes, Eugene, OR, USA) and the Zeiss Axiovert microscope (Carl Zeiss, Oberkochen, Germany) with an LSM 510 confocal attachment. In addition, Ca^{2+} sparks were assessed using the fluorescent dye fluo-4 AM (Molecular Probes) and the same confocal microscope as previously described.\(^28\)

2.2 Statistical analysis
Statistical comparison of data was performed using one-way analysis of variance followed by the Bonferroni post hoc test for individual significant differences, or Student’s t-test where appropriate. All statistical analyses were performed using Prism 4 software (Graph-Pad Software, Inc.) and \(P < 0.05\) was considered significant. Data are expressed as mean ± SEM (\(n\)), where \(n\) is the number of cells unless otherwise specified. All experiments were performed using a minimum of four animals, unless otherwise stated.

3. Results
3.1 Heart rate
Average HR was equal between the sham-operated group and the HF group (\(P > 0.05\), \(\sim 330\) b.p.m.), and, as expected for the doses employed, Iva and Met therapy caused equal HRR (\(\sim 20\%\)) in failing hearts, at all-time-points (Figure 1A). MU alone also caused HRR (\(\sim 20\%\)) at all-time-points (Figure 1B). Iva and Met therapy caused further HRR during MU, but mean HR was lower in Iva-treated (\(\sim 25\%\) HRR) compared with MU alone, than the Met-treated group during MU (\(\sim 15\%\) HRR) (Figure 1B and C).

3.2 Myocardial fibrosis
HF was associated with increased myocardial fibrosis (Figure 2). The extent of fibrosis was similar in the HF group and the MUHF group. Iva reduced levels of fibrosis during MU, whereas Met induced a small but significant increase in fibrosis during MU (Figure 2). To determine whether Iva prevents or reverses fibrosis during MU, we compared the extent of fibrosis in Iva-treated animals to those from failing animals 12-week post-LCA ligation, i.e. pre-treatment. The CAF index was lower in MUHF + Iva compared with pre-treatment group, suggesting that effects of Iva were due to reversal, not prevention of fibrosis during MU (Figure 2G). Analysis of Iva-treated non-transplanted failing hearts showed similar results. Iva reversed HF-induced interstitial fibrosis, whereas Met had no effect (Figure 3).

3.3 E–C coupling
3.3.1 Cardiomyocyte contractility
HF did not affect amplitude of sarcomeric contraction, but decreased speed of sarcomeric contraction and relaxation (Figure 4B–D). Contraction amplitude was greater in the MUHF + Iva group than MUHF + Met (Figure 4B). MU alone normalized speed of contraction; the combination with either Iva or Met had no additive effect on this parameter (Table 1, Figure 4C). MU increased speed of relaxation, but this effect was antagonized by both Iva and Met (Table 1, Figure 4D).

3.3.2 Cardiomyocyte Ca^{2+} cycling, L-type Ca^{2+} current, and t-tubule density
HF was associated with deranged Ca^{2+} cycling; Ca^{2+} transient amplitude, speed of Ca^{2+} release, and SR Ca^{2+} content were all decreased (Table 1, Figure 4), whereas speed of Ca^{2+} extrusion and fractional SR Ca^{2+} release were unaffected. MU alone normalized Ca^{2+} transient amplitude, speed of Ca^{2+} release, and SR Ca^{2+} content. Addition of Iva or Met significantly enhanced the recovery of these parameters (Table 1, Figure 4F–H). MU alone had no effect on speed of Ca^{2+} extrusion, but combination with either Iva or Met caused equal slowing of a Ca^{2+} transient decline (Table 1). MU alone did not alter fractional SR Ca^{2+} release, but this parameter was enhanced to supra-sham levels by combination with either Iva or Met (Table 1).

Reduction in the SR Ca^{2+} content can be caused by increased SR Ca^{2+} leak secondary to altered RyR function.\(^29\) Ca^{2+} sparks are a measure of diastolic SR Ca^{2+} release and are complex entities that remain only partially understood.\(^30\) Ca^{2+} spark frequency was increased in HF compared with the sham group ([sparks/100 µm²/s]: sham 0.80 ± 0.17 (37) vs. HF 3.00 ± 0.34 (40); \(P < 0.001\)), MU alone normalized this effect in HF myocytes ([sparks/100 µm²/s]: MUHF 0.82 ± 0.13 (36) vs. HF 3.00 ± 0.34 (40); \(P < 0.001\)). Neither drug affected this recovery ([sparks/100 µm²/s]: MUHF + Iva 1.61 ± 0.37 (24) vs. MUHF + Met (30) 1.70 ± 0.26; \(P > 0.05\), both \(P > 0.05\) vs. MUHF). HF induced depression of L-type Ca^{2+} current (Figure 5A) and was associated with reduced t-tubule density (Figure 5B). MU normalized both these parameters, and this effect was maintained by Iva but abolished by Met (Figure 5A and B).

A similar effect was observed in non-transplanted failing hearts: Iva normalized L-type Ca^{2+} current and t-tubule density, whereas Met had no effect (Figure 6A and B).

3.4 Myocardial atrophy
LCA ligation produced cardiac hypertrophy with an increase in both heart weight (HW) and cardiomyocyte volume (Figure 7). MU of failing hearts induced a reduction in whole-heart/cell size (myocardial atrophy); both HW and cardiomyocyte volumes falling below sham values (Figure 7). Iva therapy had no effect on MU-induced myocardial atrophy, whereas Met prevented atrophy, with no difference in mean HWs or cardiomyocyte volumes compared with the sham group.

4. Discussion
This study investigated the effects of Iva on three myocardial factors that are likely to oppose functional recovery during MU: fibrosis, derangement of E–C coupling, and atrophy. The β-blocker Met was also studied for comparison. Our results show that Iva reverses HF-induced myocardial fibrosis during MU. In addition, Iva maintains normalization of L-type Ca^{2+} current and t-tubule density, and
Figure 1  Average HR of Iva-treated and Met-treated failing hearts (A) and transplanted failing hearts (B) measured using telemetry devices. Both treatments reduced HR equally by \( \sim 20\% \) at all-time-points in failing hearts. In transplanted hearts, Iva caused slightly greater HRR than Met. \( ***p < 0.001 \) vs. HF, \( {***}p < 0.001 \) vs. MUHF and \( **p < 0.01 \) vs. MUHF + Iva, all groups (\( n = 4 \)). The percentage decrease in HR caused by Iva and Met is shown (C) Iva caused \( \sim 25\% \) and Met \( \sim 15\% \) HRR during MU \( **p < 0.01 \) and \( ***p < 0.001 \).

Figure 2  The effect of Iva and Met on interstitial fibrosis during MU. LV free wall sections from sham (A), HF (B), MUHF (C), Iva-treated (D) and Met-treated (E) animals were stained with picrosirius red. CAF was increased in HF (F). CAF was similar in the MUHF and the HF group, but significantly reduced by Iva (F). CAF in the MUHF + Iva group was lower than in HF animals 12-week post-LAD ligation (pre-treatment), suggesting that Iva reversed fibrosis during unloading (G) \( **p < 0.01 \) and \( ***p < 0.001 \). Data acquired from a minimum of 60 ventricular sections per group.
increases SR Ca\(^{2+}\) content and Ca\(^{2+}\) transient amplitude during MU. In contrast, Met antagonizes the normalization of L-type Ca\(^{2+}\) current and t-tubule density, but also enhances the recovery of SR Ca\(^{2+}\) content and Ca\(^{2+}\) transient amplitude. Sarcomeric contraction amplitude was also greater in the Iva-treated group. Finally, Iva did not prevent MU-induced myocardial atrophy, an effect that was observed with Met treatment. These results suggest that Iva may prove valuable in preventing LVAD-induced negative remodelling of the extracellular matrix (ECM) and E–C coupling, and Met in attenuating LVAD-induced myocardial atrophy.

4.1 Myocardial fibrosis
In agreement with previous studies, Iva therapy attenuated HF-induced interstitial fibrosis,\(^{18,19}\) whereas Met showed no effect. Effect of Met on fibrosis is unclear, with animal studies showing reduction,\(^{31–33}\) and others no effect.\(^{34,35}\) Metoprolol’s anti-fibrotic effects were generally associated with longer treatment durations, and the comparatively short treatment duration used in this study may explain our results. Here, we show for the first time that Iva therapy reduced fibrosis during MU and to below pre-treatment values, suggesting that reversal, not prevention, of fibrosis had occurred. This has clinical implications in that it suggests that even late administration of Iva could be of benefit. Prevention of myocardial fibrosis is a proposed therapeutic target in maximizing functional recovery during LVAD support,\(^{6}\) with elevated levels of cardiac fibrosis correlating with reduced likelihood of recovery.\(^{11,36}\) LVAD therapy alters myocardial fibrosis and collagen composition, but quantitative results are conflicting; studies reveal both increases\(^{36–38}\) and decreased myocardial fibrosis,\(^{39,40}\) and even a biphasic response following prolonged MU, i.e. ECM expansion followed by subsequent regression.\(^{31}\) Such differences are thought to arise due to the marked heterogeneity between studies with regard to HF aetiology, duration of MU, pharmacotherapy, and fibrosis quantification techniques.\(^{42}\)

In our study, MU of failing hearts did not significantly alter the CAF index. Interestingly, Iva continued to reverse fibrosis during MU, whereas Met produced a small increase in this parameter. Thus far, the anti-fibrotic effects of Iva were believed to be secondary, at least in part, to direct/indirect amelioration of heightened renin–angiotensin–aldosterone system, as well as adrenergic axis activation.\(^{18,19}\) However, our results do not support this hypothesis, as, in our experimental setting, the neurohormonal environment is neutral, provided by a healthy recipient animal. This suggests that Iva may be useful in promoting positive ECM remodelling during LVAD therapy, during which normalization of the neurohumoral environment occurs,\(^{4,43}\) and potentially acts in synergy with proven agents such as ACE-inhibitors.\(^{6,10}\) I\(_v\) channels present in ventricular myocytes, known to be mislocalized or up regulated in HF,\(^{44}\) represent a potential direct route through which Iva may have induced this effect.

4.2 E–C coupling
MU restored deranged E–C coupling, normalizing Ca\(^{2+}\) transient amplitude, speed of Ca\(^{2+}\) release and sarcomeric contraction, SR Ca\(^{2+}\) content, Ca\(^{2+}\) spark frequency, L-type Ca\(^{2+}\) current, and t-tubule density. LVAD therapy is known to increase SR Ca\(^{2+}\) content,\(^{45}\) L-type Ca\(^{2+}\) current fast inactivation, and Ca\(^{2+}\) transient amplitude,\(^{45}\) such changes correlating with improved myocyte contractility and device explantability.\(^{46}\) Mechanisms underlying such actions are unclear, but LVAD therapy is known to enhance SERCA 2A expression\(^{47}\) and stabilize RyR function.\(^{48}\) In this study, Iva enhanced MU-induced recovery of SR Ca\(^{2+}\) content and Ca\(^{2+}\) transient amplitude, parameters predictive of functional recovery,\(^{46}\) and a similar action was seen with Met, suggesting both drugs may be beneficial in enhancing functional recovery during LVAD support. The exact underlying mechanisms require further definition. The precise role of RyR function is unclear as MU-induced normalization of Ca\(^{2+}\) spark frequency was unaffected by either drug, despite an increase in the SR Ca\(^{2+}\) content. Overall, Iva was associated with more favourable effects than Met, showing greater contraction amplitude, along with the maintenance of L-type Ca\(^{2+}\) current and t-tubule

Figure 3 The effect of Iva and Met on interstitial fibrosis in non-transplanted failing hearts. LV free wall sections from sham (A), HF (B), Iva-treated (C) and Met-treated (D) animals were stained with picrosirius red. CAF was increased in HF and normalized by Iva (E). CAF in the Iva-treated group was lower than in HF animals 12-week post-LAD ligation (pre-treatment), suggesting that the reversal of fibrosis had occurred (F) ***p < 0.001. Data acquired from a minimum of 60 ventricular sections per group.
### Table 1: Contractility and Ca^{2+} handling data (16 weeks after LCA ligation)

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>HF</th>
<th>MUHF</th>
<th>MUHF + Iva</th>
<th>MUHF + Met</th>
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<tr>
<td><strong>1 Hz sarcomere shortenings</strong></td>
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<tr>
<td>Amplitude (μm)</td>
<td>0.15 ± 0.01 (50)</td>
<td>0.15 ± 0.005 (35)</td>
<td>0.14 ± 0.005 (50)</td>
<td>0.16 ± 0.01 (54)</td>
<td>0.14 ± 0.01 (47)</td>
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<tr>
<td>Time to 90% peak (ms)</td>
<td>60.2 ± 2.5 (50)</td>
<td>70.1 ± 2.1 (35)***</td>
<td>60.3 ± 1.9 (50)^^^</td>
<td>57.1 ± 2.2 (55)^^^</td>
<td>57.1 ± 2.2 (45)^^^</td>
</tr>
<tr>
<td>Speed of relaxation (μm s⁻¹)</td>
<td>19.8 ± 2.0 (48)</td>
<td>13.4 ± 2.4 (34)**</td>
<td>15.9 ± 1.6 (50)^</td>
<td>12.3 ± 1.8 (53)**</td>
<td>13.6 ± 1.9 (45)^**</td>
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<td><strong>1 Hz Ca^{2+} transient</strong></td>
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<tr>
<td>Amplitude (ratio units)</td>
<td>0.17 ± 0.01 (45)</td>
<td>0.14 ± 0.001 (35)^*</td>
<td>0.18 ± 0.005 (45)^^^</td>
<td>0.24 ± 0.01 (50)^^^</td>
<td>0.23 ± 0.01 (40)^^^</td>
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<td>Time to 90% peak (ms)</td>
<td>18.0 ± 1.1 (50)</td>
<td>24.3 ± 1.4 (35)^***</td>
<td>17.8 ± 0.8 (50)^^^</td>
<td>15.6 ± 0.6 (50)^^^</td>
<td>17.1 ± 0.6 (40)^^^</td>
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<tr>
<td>Speed of Ca^{2+} release (ratio unit⁻¹)</td>
<td>12.3 ± 0.4 (50)</td>
<td>13.1 ± 0.3 (35)</td>
<td>11.7 ± 0.4 (50)</td>
<td>10.6 ± 0.3 (50)^*</td>
<td>10.3 ± 0.4 (40)^*</td>
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<td><strong>Caffeine-induced Ca^{2+} transient</strong></td>
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<td>SR Ca^{2+} content (ratio units)</td>
<td>0.21 ± 0.01 (40)</td>
<td>0.16 ± 0.01 (28)^*</td>
<td>0.23 ± 0.01 (45)^^^</td>
<td>0.28 ± 0.01 (45)^^^</td>
<td>0.30 ± 0.01 (40)^^^</td>
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<tr>
<td>Fractional release of SR (%)</td>
<td>81.4 ± 2.1 (40)</td>
<td>80.5 ± 3.4 (28)</td>
<td>84.5 ± 2.2 (40)</td>
<td>88.5 ± 3.1 (40)^* $</td>
<td>87.1 ± 2.1 (35)^* $</td>
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<td>Contribution to Ca^{2+} decay (%)</td>
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<td>NCX</td>
<td>7.1 ± 0.6 (28)</td>
<td>7.6 ± 0.7 (40)</td>
<td>8.5 ± 0.6 (45)^*</td>
<td>9.9 ± 0.5 (45)^* $</td>
<td>9.8 ± 0.5 (28)^* $</td>
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<td>SERCA</td>
<td>92.9 ± 1.1 (28)</td>
<td>92.4 ± 0.7 (40)</td>
<td>91.5 ± 0.4 (45)^*</td>
<td>90.1 ± 0.4 (45)^* $</td>
<td>90.2 ± 1.3 (30)^* $</td>
</tr>
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*p < 0.05, **p < 0.01, ***p < 0.001 vs. sham.
^*p < 0.05, ^^p < 0.01, ^^^p < 0.001 vs. HF.
$P < 0.05.
$^p < 0.001 vs. MUHF.
$##p < 0.001 vs. MUHF + Iva.
**Figure 5** MU-induced normalization of depressed L-type Ca\(^{2+}\) current (A) and t-tubule density (measured by % Di-8-Anepps-stained area) (B). Iva did not alter these effects, whereas Met abolished them. *P < 0.05, **P < 0.01, and ***P < 0.001. Representative Di-8-Anepps-stained cells from sham (C), HF (D), MUHF (E), MUHF + Iva (F), and MUHF + Met (G) groups.

**Figure 6** HF-induced depression of L-type Ca\(^{2+}\) current (A) and t-tubule density (measured by % Di-8-Anepps-stained area) (B). Iva alone normalized these parameters, whereas Met had no effect. ***P < 0.001. Representative Di-8-Anepps-stained cells from sham (C), HF (D), HF + Iva (E), and HF + Met (F) groups.
density recovery. T-tubule disruption uncouples the normally tight relationship between L-type Ca\(^{2+}\) channels and RyRs, reducing E–C coupling efficiency.\(^{30}\) Iva therapy, but not Met, normalized t-tubule density and L-type Ca\(^{2+}\) current in non-transplanted failing hearts, possibly improving such coupling, and represents a novel action, that may have contributed to the improved whole-heart contractile function demonstrated in our, as well as previous rodent HF studies.\(^{17-20}\) Despite several beneficial effects during MU, the negative lusitropy and slowing of Ca\(^{2+}\) extrusion during MU highlight potentially unwanted effects of Iva and Met that require further investigation.

4.3 Myocardial atrophy

LVAD therapy causes a regression of cardiac and myocyte hypertrophy.\(^{49}\) The extent of this regression correlates with the duration of support.\(^{39}\) Whether prolonged clinical LVAD support brings about myocyte atrophy, the decrease in the myocyte size to subnormal values (not necessarily dysfunctional), is unclear and remains controversial.\(^{50}\)

Along with myocardial fibrosis, prevention of myocardial atrophy during prolonged LVAD therapy is a proposed therapeutic target for enhancing BTR.\(^{5,24}\) but the precise importance of size in relation to function remains unproven. Clenbuterol, in combination with aggressive medical therapy, has enhanced BTR rates as part of the Harefield protocol,\(^{8}\) but evidence supporting an anti-atrophic action is minimal.\(^{46}\) Experimentally, HATx has been convincingly demonstrated to induce myocardial atrophy.\(^{24,25,51}\) We show that Iva did not prevent MU-induced atrophy, but surprisingly Met proved successful. Hitherto, no pharmacological agent has proved effective in attenuating myocardial atrophy, with clenbuterol only limiting regression of hypertrophy of failing rat hearts, after a short 1-week period of MU.\(^{23}\) when atrophic remodelling is submaximal.\(^{24,25}\) Partial haemodynamic loading\(^{51,52}\) and reloading\(^{53}\) of unloaded rodent LVs attenuates atrophy, re-iterating the importance of load in regulating cardiac mass.\(^{25,51}\) Such loading, potentially brought about by HRR and subsequent augmentation of LV filling, may have favoured anti-atrophic actions of Met, but the lack of the effect of Iva, despite a slightly larger HRR, makes this mechanism unlikely.

4.4 In vivo whole-heart function and heart rate

Echocardiographic assessment of non-transplanted failing hearts confirmed that Iva significantly improves LVEF and LVFS in HF as previously shown,\(^{17-20}\) with Met showing a minimal effect.\(^{54}\) (Supplementary material online, Table S1). The measurement of in vivo whole-heart contractile function in abdominally transplanted hearts is problematic, and the reliability and value of standard echocardiography, MRI, and pressure–volume-derived functional parameters are questionable.\(^{55}\) As such, in vivo whole-heart function was not assessed in this study, and in the view of the distinct treatment effects on
myocardial atrophy and fibrosis would have provided additional valuable information.

The isolated HR effects of MU remain unstudied in both the experimental, and clinical setting. In this study, transplanted HR was lower than non-transplanted, most likely due to cardiac denervation, absence of heightened sympathetico-adrenergic stimulation, and reduced stretch-induced modulation of SAN automaticity. HR is a critical regulator of myocardial oxygen delivery and consumption. HRR prolongs diastolic duration, improves coronary flow, and enhances myocardial energetic balance, while concurrently reducing myocardial oxygen consumption. Therefore, it is reasonable to believe MU-induced HRR contributed to myocyte recovery. In non-transplanted failing hearts, HRR via Iva is associated with improved cardiac metabolism and coronary reserve, and induces angiogenesis. Likewise, these mechanisms may have contributed to the enhancement of Iva of the myocyte recovery, and the reversal of myocardial fibrosis during MU. The exact size and nature of this HRR-dependent contribution needs to be established, as the prospect that Iva’s actions extend beyond that of pure HRR has been raised. Likewise, the isolated contribution of HRR to effects of Met also requires definition.

Drug doses used in this study were based on previous work showing equal HRR in non-transplanted failing rodent hearts. The unanticipated difference in HRR between drugs in transplanted hearts prevented exact analysis of drug effects independent of HR, but we feel is unlikely to underpin the distinct qualitative differences in drug effects shown by this study. This prospect is reinforced by the markedly different drug effects exerted on non-transplanted failing hearts shown in this study, and by others, during which equivalent HRR was achieved.

4.5 Heterotopic abdominal heart transplantation as a model of LVAD support

HATx is a well-established small animal model to produce profound volume unloading of the LV, and was therefore chosen as the model to mimic the MU produced by LVAD support. Reduced but intermittent aortic valve opening is seen with both, suggesting partial pressure unloading of the LV also occurs. However, several differences between HATx and LVAD support are recognized and need to be considered. The transplanted heart is denervated, whereas the LVAD-implanted heart is not. The LV of the transplanted heart empties through the aortic valve alone, whereas that of the LVAD-implanted heart ejects through both the aortic valve and/or the LVAD outflow cannula. In addition, a more gradual neurohumoral amelioration occurs during LVAD support. Ideally, the study of the effects of ivabradine during LVAD support in a large animal model would be the next desirable step. In addition, the effects of ivabradine on mechanically unloaded non-failing hearts were not studied as part of this paper but this would be of great value with regard to further elucidation of ivabradine’s mechanisms of action and warrants further future studies.

In conclusion, this study shows for the first time that Iva exerts beneficial structural and E–C coupling remodelling effects during MU. Iva (i) reversed myocardial fibrosis and (ii) enhanced restoration of deranged E–C coupling, displaying more favourable effects than that of Met. MU-induced myocardial atrophy was unaffected by Iva but was prevented by Met. These results suggest that Iva may prove effective in preventing LVAD-induced negative remodelling of the ECM and E–C coupling, and therefore in enhancing functional myocardial recovery in HF patients receiving LVAD support.

Supplementary material
Supplementary material is available at Cardiovascular Research online.

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References
4. Klotz S, Jan Danzer AH, Burkhoff D. Impact of left ventricular assist device (LVAD) support on the cardiac reverse remodeling process. Prog Biophys Mol Biol 2008;97:
Ivabradine and mechanical unloading


