Alterations in Sarcoplasmic Reticulum Gene
Expression in Human Heart Failure
A Possible Mechanism for Alterations in Systolic and
Diastolic Properties of the Failing Myocardium

Masashi Arai, Norman R. Alpert, David H. MacLennan, Paul Barton, and Muthu Periasamy

Recent studies have shown that intracellular Ca\(^{2+}\) handling is abnormal in the myocardium of patients with end-stage heart failure. Muscles from the failing hearts showed a prolonged Ca\(^{2+}\) transient and a diminished capacity to restore a low resting Ca\(^{2+}\) level during diastole. Accordingly, we examined whether this defect in Ca\(^{2+}\) transport function is due to alterations in sarcoplasmic reticulum gene expression. We determined the messenger RNA (mRNA) levels of sarcoplasmic reticulum Ca\(^{2+}\) transport proteins in failing human hearts from 17 cardiac transplant recipients with a diagnosis of dilated cardiomyopathy, primary pulmonary hypertension, or ischemic heart disease. The expression levels of each mRNA were compared with each other and then correlated with that of atrial natriuretic factor (ANF) mRNA in the failing ventricle. The mRNA levels for the calcium release channel (ryanodine receptor, RYR2), Ca\(^{2+}\) uptake pump (Ca\(^{2+}\)-ATPase, SERCA2a isoform), and phospholamban differed significantly between heart samples but showed an inverse relation with that of ventricular ANF mRNA. In contrast, calsequestrin mRNA levels remained unchanged in these failing hearts. In addition, \(\beta\)-myosin and \(\alpha\)-cardiac actin mRNA levels also showed an inverse relation with ANF mRNA levels. These changes were observed in both right and left ventricles of hearts with congestive heart failure due to dilated cardiomyopathy, primary pulmonary hypertension, or ischemic heart disease. The results are consistent with the hypothesis that abnormal calcium handling in the sarcoplasmic reticulum of failing hearts is due to the altered expression of the genes encoding sarcoplasmic reticulum proteins. (Circulation Research 1993;72:463–469)

KEY WORDS • ryanodine receptor • phospholamban • calsequestrin • sarcoplasmic reticulum • Ca\(^{2+}\)-ATPase

Heart failure is a low cardiac output syndrome characterized by both systolic and diastolic dysfunction. The velocity and extent of ventricular contraction and the rate of pressure development are decreased in heart failure.\(^1,2,3\) The left ventricular (LV) relaxation rates, assessed by maximum rates of LV pressure decline, and the mean velocity of circumferential fiber length shortening in early diastole are also decreased, suggesting an impairment of early diastolic LV relaxation.\(^4\) The contraction and relaxation of cardiocytes are regulated by intracellular calcium (Ca\(^{2+}\)) concentrations, which, in turn, are controlled primarily by the release and reuptake of Ca\(^{2+}\) by the sarcoplasmic reticulum (SR). Accordingly, defects in Ca\(^{2+}\) release and Ca\(^{2+}\) uptake functions of the SR could result in the systolic and diastolic dysfunctions seen in heart failure.

In recent years, the major SR proteins controlling Ca\(^{2+}\) uptake, storage, and release have been isolated, and sequencing of complementary DNA (cDNA) encoding them has provided the deduced amino acid sequences.\(^5\) The contraction of cardiac myocytes is triggered by Ca\(^{2+}\) release from the SR through a calcium release channel, also referred to as the ryanodine receptor (RYR).\(^6\) Two distinct isoforms of Ca\(^{2+}\) release channel (RYR) have been described by cDNA cloning,\(^7–10\) but only the RYR2 isoform is expressed in cardiac tissues.\(^10,11\) Muscle relaxation is initiated by ATP-dependent transport of Ca\(^{2+}\) uptake into the SR. Five distinct Ca\(^{2+}\)-ATPase isoforms have been identified,\(^5\) the cardiac/slow-twitch isofrom (SERCA2a) being the major Ca\(^{2+}\)-ATPase expressed in cardiac tissue.\(^11,13\) The function of SERCA2a is inhibited by its interaction with a regulatory protein, phospholamban, but inhibition is relieved by both cyclic AMP (cAMP) and calmodulin-dependent phosphorylation of phospholamban.\(^14\) Phospholamban is encoded by a single gene, and the same protein is expressed in cardiac and slow-twitch skeletal muscle tissues.\(^15\) Calsequestrin, a high-capacity,
moderate-affinity Ca\(^{2+}\) binding protein, is localized in the lumen of the SR and is the major determinant of the Ca\(^{2+}\) storage capacity of SR. Two distinct isosforms of calsoquestrin have been identified,\(^1^6\) -\(^1^8\) the skeletal muscle isoform being expressed in both fast- and slow-twitch fibers and the cardiac isoform being expressed exclusively in the cardiac muscle.\(^1^7\) -\(^1^8\)

Several studies have described alterations of SR function in human heart failure. Intracellular Ca\(^{2+}\) measurements using aequorin showed that Ca\(^{2+}\) transients in muscles from failing human hearts were markedly prolonged in both in the Ca\(^{2+}\) release and uptake phases.\(^1^9\) -\(^2^0\) Ca\(^{2+}\) uptake rates were reported to be diminished by 50% in right ventricular biopsy samples from failing hearts,\(^2^1\) and messenger RNA (mRNA) for Ca\(^{2+}\)-ATPase was shown to be decreased by 48% in LV specimens from patients undergoing heart transplantation.\(^2^2\) The expression of phospholamban mRNA was also markedly decreased in failing hearts.\(^2^3\) On the other hand, Movsesian and coworkers\(^2^4\) -\(^2^5\) reported that in isolated SR vesicles, the Ca\(^{2+}\) uptake rates and phospholamban-mediated modulation of Ca\(^{2+}\) uptake were not significantly different between ventricular samples from patients with idiopathic dilated cardiomyopathy and control hearts. Thus, the role of SR function in human heart failure warrants further investigation. In the present study, we have investigated the molecular basis of pathophysiological alterations of SR function in human heart failure. Because it was difficult to obtain normal human hearts, we compared failing hearts expressing different levels of ventricular atrial natriuretic factor (ANF) mRNA. We determined the expression level of individual mRNAs encoding the SR Ca\(^{2+}\) release channel, Ca\(^{2+}\)-ATPase, calsoquestrin, and phospholamban, as well as actin and myosin, in heart failure and assessed the extent of coordinate regulation among them. The expression levels of these mRNAs were then correlated with ventricular ANF mRNA level.

**Materials and Methods**

**Human Heart Samples**

Right ventricular and LV tissues from end-stage failing hearts were obtained and examined from 17 cardiac transplant recipients: five patients with primary pulmonary hypertension, four patients with ischemic heart disease, and eight patients with dilated cardiomyopathy. These tissues were obtained from Harefield Hospital, London, and informed consent was obtained from all patients. Patients' ages ranged from 3 to 63 years (mean age, 38 years). After removal of the heart from the thoracic cavity, specimens were excised separately from right ventricular and LV free wall. Effort was made to avoid scarred connective tissue or other nonmyocardial constituents when taking samples. These tissues were frozen immediately and stored in liquid nitrogen. Clinical and hemodynamic data for each patient are summarized in Table 1.

**RNA Isolation and Northern Blot Analysis**

Total cellular RNA was isolated by use of the guanidine thiocyanate procedure.\(^1^1\) Total RNA (15 \(\mu\)g) was denatured at 65°C for 5 minutes, fractionated on a 0.8% agarose gel containing 2.2 M formaldehyde, transferred onto a nitrocellulose membrane (Schleicher & Schuell, Inc., Keene, N.H.), and hybridized to random primed cDNA probes. The membranes were washed with 1× standard saline citrate and 0.1% sodium dodecyl sulfate at 55°C for 30 minutes, air dried, and exposed to Kodak

<table>
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<th>Case</th>
<th>Age</th>
<th>Diagnosis</th>
<th>PAWP or LVEDP (mm Hg)</th>
<th>LVDD/LVDS (mm)</th>
<th>EF (%)</th>
<th>ANF mRNA (arbitrary units)</th>
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<td>12</td>
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<tr>
<td>(r)</td>
<td>...</td>
<td>...</td>
<td>0.72*</td>
<td>0.45/0.58</td>
<td>0.49</td>
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</table>

ANF, atrial natriuretic factor; mRNA, messenger RNA; PAWP, pulmonary artery wedge pressure; LVEDP, left ventricular end-diastolic pressure; LVDD/LVDS, left ventricular diameter in diastole (d) and in systole (s); EF, ejection fraction; PPH, primary pulmonary hypertension; IHD, ischemic heart disease; DCM, dilated cardiomyopathy. \(r\), Spearman's rank correlation coefficient between ANF mRNA level.

\(^{*}\) \(p<0.05\).
X-Omat AR film for 48 hours using an intensifying screen at −70°C. The expression level of each mRNA was quantified directly from the nitrocellulose membrane by use of a Betascope 603 blot analyzer (Betagen Corp.).

**DNA Probes**

The following cDNA probes were used for Northern blot analysis: 1) rabbit cardiac RYR cDNA, 7,984–10,237; 2) human cardiac Ca\(^{2+}\)-ATPase cDNA, nucleotides 774–1,449; 3) rabbit phospholamban cDNA, nucleotides 177–1,191; 4) rabbit cardiac calsequestrin cDNA, nucleotides −85–2,416; 5) human α-myosin heavy chain (MHC), nucleotides −81–8, derived by polymerase chain reaction (PCR) of the 5′ nontranslated region; 6) human β-MHC, 104 base pairs of 3′ noncoding region; 7) human α-cardiac actin, 171 base pairs of 3′ noncoding region; and α-skeletal actin, 136 base pairs of 3′ noncoding region; 8) human ANF cDNA nucleotides −11–749; and 9) chicken glyceraldehyde-3-phosphate dehydrogenase (GAPDH), nucleotides −30–1,092. GAPDH mRNA levels were used as an internal standard for the variations in sample loading and blotting efficiency of RNA. The expression level of each mRNA was divided by the GAPDH mRNA value for statistical analyses, since the GAPDH mRNA level was proportional to the intensity of 28S and 18S ribosomal RNA on ethidium bromide staining.

**Statistical Analysis**

For the estimation of correlation between each parameter, Spearman’s rank correlation coefficient (r) was calculated. The test was considered significant at p<0.05.

**Results**

**Atrial Natriuretic Factor mRNA Expression in Failing Human Ventricles**

ANF mRNA is not normally detectable in nonfailing ventricular tissue. But several studies have shown...
that ventricular ANF mRNA level increased with hyper trophy and heart failure in both animal and human hearts.\textsuperscript{32-35} In this study, ANF mRNA was detectable in all failing human hearts; its expression level varied more than 100-fold between heart samples (Table 1). In addition, there was no obvious correlation between the type of heart disease and ANF mRNA (Table 1) or between the left and right ventricles (data not shown).

The Expression Levels of mRNA for the Ca\textsuperscript{2+} Release Channel (RYR2), the Ca\textsuperscript{2+}-ATPase (SERCA2), and Phospholamban Were Inversely Related to That of Ventricular ANF mRNA in Failing Human Ventricles

The expression levels of mRNAs encoding several Ca\textsuperscript{2+} transport proteins were examined using total RNA from hearts exhibiting various degrees of failure. The mRNA levels of individual SR proteins from 34 samples are shown in Figure 1. Their expression level varied severalfold among samples examined. For example, the mean values of 10 samples with lowest expression in RYR2, SERCA2, and phospholamban were 27\%, 37\%, and 34\% of the mean level of 10 samples with highest expression, respectively. Normal human hearts were not available, so we determined the correlation between individual Ca\textsuperscript{2+} transport protein mRNA levels with that of ANF mRNA levels.\textsuperscript{32-35} Figure 2 shows a correlation between Ca\textsuperscript{2+}-ATPase mRNA level and ANF mRNA levels in all samples examined. Northern blot analysis of mRNA samples from five representative hearts with different degrees of failure as indicated by levels of ventricular ANF mRNA is shown in Figure 3. The ventricular sample with highest ANF mRNA level (ANF mRNA, 127.5 units) is shown in lane 3; moderate levels of ANF mRNA are seen in lanes 1 and 2 (lane 1, 15.2 units; lane 2, 30.6 units); and representative samples of the lowest ANF mRNA levels are shown in lanes 4 and 5 (lane 4, 4.9 units; lane 5, 7.2 units). The expression levels of RYR2 mRNA, SERCA2 mRNA, and phospholamban were inversely related to the ANF mRNA level, with the lowest levels found in lane 3, the highest levels in lanes 4 and 5, and intermediate levels in lanes 1 and 2. Thus, as the expression level of ANF mRNA was increased, the expression of the mRNA for the three calcium cycling proteins was decreased (Figure 3). All three mRNAs showed a significant inverse correlation with that of ANF mRNA in all 34 samples examined \textbullet{} (between ANF mRNA and RYR2, \(r=-0.65, p<0.01\); SERCA2, \(r=-0.78, p<0.01\); and phospholamban, \(r=-0.84, p<0.01\)) (Table 2). The mRNAs encoding SR proteins also showed significant positive correlations with each other, suggesting that their expression was coordinately regulated (RYR2 versus Ca\textsuperscript{2+}-ATPase, \(r=0.65, p<0.01\); SERCA2 versus phospholamban, \(r=0.82, p<0.01\); phospholamban versus RYR2, \(r=0.42,

\textbf{TABLE 2. Correlation Coefficient Between ANF mRNA and Ca\textsuperscript{2+} Regulatory Protein mRNA Level}

<table>
<thead>
<tr>
<th>mRNA</th>
<th>RYR2</th>
<th>SERCA2</th>
<th>PHLB</th>
<th>C-CSQ</th>
<th>(\beta)-MHC</th>
<th>(\alpha)-C-actin</th>
<th>(\alpha)-Sk-actin</th>
</tr>
</thead>
<tbody>
<tr>
<td>All ((n=34))</td>
<td>(-0.65^*)</td>
<td>(-0.78^*)</td>
<td>(-0.84^*)</td>
<td>(-0.32)</td>
<td>(-0.76^*)</td>
<td>(-0.50^*)</td>
<td>(0.04)</td>
</tr>
<tr>
<td>LV ((n=17))</td>
<td>(-0.69^{**})</td>
<td>(-0.67^*)</td>
<td>(-0.86^*)</td>
<td>(-0.52^{**})</td>
<td>(-0.73^*)</td>
<td>(-0.12)</td>
<td>(-0.33)</td>
</tr>
<tr>
<td>RV ((n=17))</td>
<td>(-0.54^{**})</td>
<td>(-0.71^*)</td>
<td>(-0.52^{**})</td>
<td>(0.12)</td>
<td>(-0.47)</td>
<td>(-0.51^{**})</td>
<td>(-0.38)</td>
</tr>
<tr>
<td>PPH ((n=10))</td>
<td>(-0.90^*)</td>
<td>(-0.88^*)</td>
<td>(-0.62)</td>
<td>(-0.10)</td>
<td>(-0.05)</td>
<td>(-0.65^{**})</td>
<td>(-0.05)</td>
</tr>
<tr>
<td>IHD ((n=8))</td>
<td>(-0.43)</td>
<td>(-0.48)</td>
<td>(-0.83^{**})</td>
<td>(0.21)</td>
<td>(-0.79^{**})</td>
<td>(-0.07)</td>
<td>(-0.07)</td>
</tr>
<tr>
<td>DCM ((n=16))</td>
<td>(-0.61^{**})</td>
<td>(-0.73^*)</td>
<td>(-0.77^*)</td>
<td>(-0.39)</td>
<td>(-0.83^*)</td>
<td>(-0.37)</td>
<td>(0.07)</td>
</tr>
</tbody>
</table>

ANF, atrial natriuretic factor; mRNA, messenger RNA; RYR2, cardiac ryanodine receptor; SERCA2, slow-twitch cardiac Ca\textsuperscript{2+}-ATPase; PHLB, phospholamban; C-CSQ, cardiac calsequestrin; MHC, myosin heavy chain; \(\alpha\)-C-actin, \(\alpha\)-cardiac actin; \(\alpha\)-Sk-actin, \(\alpha\)-skeletal actin; LV and RV, left and right ventricular samples including all PPH (primary pulmonary hypertension), IHD (ischemic heart disease), and DCM (dilated cardiomyopathy) patients. PPH, IHD, DCM: samples from both RV and LV in each disease. \(^*p<0.01; ^{**}p<0.05\).
p<0.05). On the contrary, the expression of cardiac calsequestrin mRNA did not change significantly and failed to show a correlation with that of ANF mRNA (r=-0.32, NS) (Table 2). Similar changes were noted when samples were analyzed from the left or the right ventricle and in hearts with different disease histories (Table 2).

**Figure 3.** Northern blot analysis showing messenger RNA (mRNA) levels of different sarcoplasmic reticulum proteins in failing human hearts. Total RNA (15 μg) from left ventricles from five dilated cardiomyopathy patients was blotted and hybridized as described in "Materials and Methods" with cardiac ryanodine receptor (C-RYR), sarcoplasmic reticulum Ca²⁺-ATPase, phospholamban (PHLB), cardiac calsequestrin (C-CSQ), atrial natriuretic factor (ANF), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probes. Lane numbers 1–5 correspond to case numbers 12, 14, 16, 11, and 13 in Table 1, respectively. Duplicate filters made from two identical RNA–agarose gels were hybridized sequentially with these probes. The estimation of GAPDH mRNA levels was used as an internal standard for correction of loading variation in each RNA sample. The bottom panel shows ethidium bromide staining of the agarose gel. rRNA, ribosomal RNA.

**Figure 4.** Northern blot analysis showing myosin and actin messenger RNA levels. Duplicate filters made from two identical RNA–agarose gels were stripped and hybridized with β-myosin heavy chain (β-MHC), α-cardiac actin (α-CActin), and α-skeletal actin (α-SkActin)–specific cDNA probes. Lane numbers 1–5 correspond to case numbers 12, 14, 16, 11, and 13 in Table 1, respectively. The bottom panel shows ethidium bromide staining of the agarose gel. rRNA, ribosomal RNA.

β-MHC and α-Actin mRNA Levels Also Showed an Inverse Relation to ANF mRNA Levels

We next examined whether expression levels of mRNAs for MHC and actin were also altered in heart failure using the same RNA samples (Figures 1 and 4). The expression levels of mRNAs encoding β-MHC and α-cardiac actin were also modified and exhibited an inverse correlation with the ANF mRNA level (r between ANF mRNA and β-MHC, -0.76, p<0.01; α-cardiac actin, -0.50, p<0.01) (Figure 4, Table 2). In contrast, the expression of α-skeletal actin mRNA remained high and did not show a correlation with ANF mRNA (r between ANF mRNA and α-skeletal actin, 0.04, NS) (Figure 4, Table 2). The expression of α-MHC mRNA was not detectable in any of the hearts examined.

**Discussion**

In this study, we report that the mRNA levels for the SR Ca²⁺ release channel, Ca²⁺-ATPase, and phospholamban are drastically altered in hearts from patients with end-stage heart failure. In addition, MHC and α-cardiac actin mRNA were also altered in failing hearts. Because normal human hearts were difficult to
obtain, we were unable to determine the absolute decrease in mRNA levels in failing hearts, but a comparison was made between failing heart samples expressing different levels of ventricular ANF mRNA. More importantly, our measurements of the mRNAs for various Ca\(^{2+}\) transport proteins could be correlated with the abnormalities in Ca\(^{2+}\) release and uptake in the SR from failing hearts.

It has been shown that increases in circulating concentrations of ANF are associated with elevated cardiac filling pressure in the human failing heart\(^{24}\) and that plasma ANF concentration is increased in New York Heart Association (NYHA) functional classes II and III (moderate and severe) heart failure patients and is highest in NYHA class IV (severest) heart failure patients.\(^{35}\) It has also been reported that the level of ventricular ANF mRNA expression is markedly increased in moderate heart failure and is highest in severe heart failure in the cardiomyopathic hamster model of congestive heart failure.\(^{32,36}\) In this study, we have also found the significant positive correlation between LV ANF mRNA level and LV end-diastolic pressure or pulmonary artery wedge pressure \((r=0.72, p<0.05)\) in 10 patients for whom we could collect clinical data. Because the number of the patients examined and the hemodynamic parameters used were limited, whether ventricular ANF mRNA is directly proportional to the severity of heart failure remains to be determined. However, the analysis from this study and previous studies\(^{32-37}\) suggests that in general, ventricular ANF mRNA level would be higher in severe heart failure than in mild heart failure.

In this study, we have shown that the expression levels of mRNA for Ca\(^{2+}\) release channel, Ca\(^{2+}\)-ATPase, and phospholamban were inversely correlated with the ANF mRNA level, suggesting that the expression of these mRNAs was decreased in severe heart failure. Gwathmey et al.\(^{19}\) and Morgan and et al.\(^{20}\) reported that muscle from failing human hearts exhibits abnormal Ca\(^{2+}\) handling, manifested as prolonged Ca\(^{2+}\) transients, which indicates a diminished capacity to restore a low resting calcium level during diastole. Furthermore, tension-independent heat measurements showed a substantial reduction in the amount of heat produced in muscle strips from the failing myocardium. Tension-independent heat is thought to result from the energy expended for Ca\(^{2+}\) transport in the muscle strips and can provide an estimate of calcium cycled per heart beat.\(^{38}\) The amount of calcium cycled was 32.2±8.17 nmol/g per beat in control hearts and 16.7±1.72 nmol/g per beat in failing hearts,\(^{39}\) indicating a \(^{-50}\%\) reduction in Ca\(^{2+}\) uptake function. Our measurements carried out at the mRNA level do not directly reflect the Ca\(^{2+}\) uptake or Ca\(^{2+}\) release function of failing myocardium. However, the results presented here suggest that decreased expression of mRNA for both Ca\(^{2+}\) release and Ca\(^{2+}\) uptake proteins of SR will lead to a decrease in the relative number of excitation–contraction coupling sites and could be responsible for abnormal Ca\(^{2+}\) handling by the SR in failing myocardium.

In this study, we have shown that Ca\(^{2+}\) release channel mRNA levels are decreased, as noted for the Ca\(^{2+}\)-ATPase in failing hearts. These results are also supported by two other recent reports on this subject.\(^{37,40}\) Our findings that the mRNA for Ca\(^{2+}\) release channels is coordinately decreased with that of Ca\(^{2+}\) pumps in failing human hearts is consistent with our earlier studies of other systems. In studies of animal models, we showed that in hyperthyroid hearts, the mRNA for Ca\(^{2+}\) release channels and Ca\(^{2+}\) pumps are coordinately increased, whereas in hypothyroidism, they are decreased.\(^{41}\) These findings indicate that a functional coordination exists between Ca\(^{2+}\) uptake and Ca\(^{2+}\) release processes. It is unclear at this time how this coordination is achieved at the gene level.

In these studies, only calsequestrin mRNA levels did not show a significant correlation with ANF mRNA levels. Previous studies indicated that the maximal Ca\(^{2+}\) binding capacity of SR is diminished in cardiomyopathic hamsters\(^{42}\) and in failing human hearts due to ischemic heart disease compared with control animals.\(^{43}\) Our data suggest that the Ca\(^{2+}\) sequestering ability of the SR is not changed in failing human hearts. Interestingly, the expression of calsequestrin is regulated independently from other SR Ca\(^{2+}\) regulatory proteins. A lack of coordinate regulation of calsequestrin expression with other SR proteins was also seen in hyperthyroid and hypothyroid rabbit hearts.\(^{41}\)

Our analysis of MHC expression suggested that β-MHC expression was decreased in hearts with severe heart failure. A true estimate of the decrease could not be made, because control hearts were unavailable. However, in these failing hearts, α-MHC mRNA was not detectable. Thus, myosin isoform switching was not a major adaptive response in these failing human hearts. Our analysis suggested that α-cardiac actin mRNA was decreased in severe heart failure, whereas α-skeletal actin mRNA maintained a high level of expression as found in normal human myocardium (K. Schwartz, personal communication). Thus, a cardiac–to–skeletal isoform transition (as found in small mammals) is not seen for actin protein.

In conclusion, our studies on SR gene expression in human failing hearts suggest that a defect exists in the Ca\(^{2+}\) regulatory properties of the SR. Thus, abnormal calcium handling resulting from a decrease in the relative number of excitation–contraction coupling sites, which is suggested by the decrease of corresponding mRNA expression, could drastically modify the force–frequency relation and myocardial performance. These observations might have important implications for diagnosis and therapy of heart failure patients.

Acknowledgments

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