Deficiency of type 1 cannabinoid receptors worsens acute heart failure induced by pressure overload in mice

Yulin Liao1*, Jianping Bin1, Masanori Asakura2, Wanling Xuan1,3, Baihe Chen1,3, Qiaobing Huang3, Dingli Xu1, Catherine Ledent4, Seiji Takashima5, and Masafumi Kitakaze1,2*

1Department of Cardiology, and Organ Failure Key Laboratory of Ministry of Education, Nanfang Hospital, Southern Medical University, 1838 Guangzhou avenue north, Guangzhou 510515, China; 2Cardiovascular Division of the Department of Medicine, National Cerebral Cardiovascular Center, 5-7-1 Fujishirodai, Suita, Osaka 565-8565, Japan; 3Department of Pathophysiology, Key Laboratory of Shock and Microcirculation Research, Southern Medical University, Guangzhou 510515, China; 4IRIBHN Université Libre de Bruxelles, Bruxelles, Belgium; and 5Molecular Cardiovascular Medicine, Osaka University Graduate School of Medicine, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan

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Aims
We investigated the influence of type one cannabinoid receptor (CB1) deficiency on acute heart failure (AHF) and the underlying mechanism. Acute heart failure syndrome is an important clinical problem because of its high morbidity and mortality rates. Activation of CB1 induces vascular dilation and reinforces the properties of morphine, long-standing therapies for AHF syndrome, but the effect of endogenous CB1 activation on AHF is largely unknown.

Methods and results
Acute heart failure mouse model characterized by hypertension and pulmonary oedema was created by using transverse aortic constriction (TAC). Mortality, echocardiography, haemodynamic, morphology, and circulatory catecholamine levels in response to TAC were evaluated in CB1 knockout (KO) and wild-type mice. Type one cannabinoid receptor KO mice had a much higher mortality rate at 1 week after TAC attributable to AHF (65 vs. 11%, \( P < 0.001 \)). One hour after TAC, CB1 KO mice had significant larger lung weight to body weight ratio (LW/BW, 14.53 ± 1.09 mg/g in KO vs. 10.42 ± 0.36 mg/g in WT, \( P < 0.01 \)) and higher plasma epinephrine levels (9720 ± 1226 pg/mL vs. 6378 ± 832 pg/mL, \( P < 0.05 \)). Pharmacological activation of CB1 reduced LW/BW in wild-type mice. Administration of epinephrine to wild-type TAC mice significantly increased left ventricular end-diastolic pressure and LW/BW, while CB1 agonists reduced the LW/BW and the plasma levels of catecholamine and increased myocardial activity of AMP-activated protein kinase.

Conclusion
Endogenous activation of CB1 in mice has cardiac protection in AHF, which is attributable to the inhibition of excessive sympathetic activation.

Keywords
Cannabinoid receptor • Acute heart failure • Catecholamine • Mortality • Mouse

Introduction
Patients with cardiovascular diseases are increasingly hospitalized due to acute heart failure syndromes (AHFS). However, traditional therapies for AHFS, such as oxygen, loop diuretics, nitrates, and morphine have significant limitations, and the mortality rate remains relatively high, indicating the need to develop more effective treatments. Cannabinoids have not only neurobehavioural, but also cardiovascular effects. Both of cannabinoids receptors 1 and 2 (CB1 and CB2, respectively) exist in the cardiovascular system. Recent published literature has addressed the influence of CB1 activation on the uncommon forms of heart failure induced by hepatic cirrhosis or doxorubicin, however, the influence of endogenous CB1 activation on the classical AHFS remains poorly understood.

Type one cannabinoid receptor agonists have been shown to exhibit a vasodilatory effect, inhibit the release of neurohormonal factors, improve myocardial energy metabolism, and suppress...
vasopressin-induced vasoconstriction. More importantly, it was reported that a CB1 antagonist increased the acute mortality rate after myocardial infarction in rats. These findings raise the possibility that CB1 signalling may play an important role in AHF.

In the present study, we hypothesized that activation of CB1 might be beneficial for AHF. To verify this hypothesis, we first established a murine model of AHF mimicking the clinic profiles of pulmonary oedema and high blood pressure, and then investigated the influence of the CB1 activity in AHF by studying the cardiac changes in CB1 knockout (KO) mice and the influence of CB1 agonists or antagonists on heart function in wild-type (WT) mice as well as the underlying mechanism.

### Methods

#### Agents

WIN 55,212-2 (WIN), and AM 251, [N-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide], were purchased from Tocris Bioscience (Ellisville, MO). 2-Arachidonyl glycerol (2-AG) was purchased from Sigma RBI. Epinephrine was purchased from Sigma Chemical Company (see Supplementary material online).

#### Transverse aortic constriction model

All procedures were performed in accordance with our institutional guidelines for animal research that conformed to the ‘Position of the

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**Figure 1** Acute heart failure induced by transverse aortic constriction in both ICR and C57 mice. (A) Representative recordings of blood pressure made simultaneously from the right and left carotid arteries. (B) Left ventricular systolic and end-diastolic pressure. (C) Maximal left ventricular pressure rise and fall rate (±dp/dt max). (D) Representative recordings of left ventricular pressure and pressure change rate. (E) Heart rate and the exponential time constant of relaxation (Tau). (F) The lung weight to body weight ratio. *P < 0.05, **P < 0.01, n = 7 in each group.
American Heart Association on Research Animal Use’ adopted by the AHA on 11 November 1984. Type one cannabinoid receptor (−/−), CB1(+/-+) or C57 mice were used. Development of mice lacking CB1 was previously described by Ledent et al. Male C57BL/6 mice (7 weeks old and weighing 20–24 g) or ICR or CB1 mice (5–7 weeks) were anaesthetized with pentobarbital sodium (50 mg/kg). To induce acute pulmonary oedema, transverse aortic constriction (TAC) was performed as described elsewhere.12 degree of aortic stenosis was controlled by the size of the banded needle which was withdrawn after aortic ligation and confirmed by the pressure gradient across the banded site (Figure 1A), which was usually >50 mmHg at the 3rd day after TAC in our laboratory.

Transsthoracic echocardiography was performed with a Sonos 4500 and a 15-6 L MHz transducer (Philips, the Netherlands) and invasive assessment of haemodynamic was carried out using a Millar catheter (see Supplementary material online).

**Measurement of catecholamine and endocannabinoids and blood cell counts**

Plasma catecholamine (epinephrine, norepinephrine, and dopamine) were measured by SRL, Inc. (Kyoto, Japan) using high-performance liquid chromatography (HPLC). Anandamide (AEA) and N-oleylethanolamine (OEA) in the heart and lung samples HPLC–tandem mass spectrometry (HPLC/MS-MS) (see Supplementary material online).

Circulating blood cell counts (red cells, leucocytes, and platelets) were measured using a Sysmex KX-21 hematology analyzer (Sysmex, Japan) (see Supplementary material online, Methods).

**Cell culture and immunoblotting**

Ventricular myocytes were isolated from neonatal rats at 2–3 days of life and cultured. Proteins were prepared from cultured cardiomyocytes or whole hearts of mice. Then immunoblotting was performed using mouse antibodies directed against phosphorylated AMP-activated protein kinase (AMPK), which recognizes the AMPK pan-subunit phosphorylated at Thr-172, or AMPK (Cell Signaling). Immunoreactive bands were visualized by the enhanced chemiluminescence method (Amersham) and then were quantified by densitometry with Scion Image software.

**Knockdown of rat type one cannabinoid receptor using siRNA and real-time PCR**

Neonatal rat cardiomyocytes at 50–70% confluence were transfected with CB1 siRNA (designed and synthesized by B-Bridge International, Inc.) with Optifect (Invitrogen Co.). SiRNA for CB1 was transfected at 66 nM at 4–6 h after plating of cells into a 60-mm culture dish. The siRNA sequences were 5′-ggg aag aug aac aag cuu a-TT (sense) and 5′-uaa gcu ugu uca ucu ucc c-TT (antisense), while the control siRNA was uag cga cua aac aca uca a-dTdT.

Real-time PCR for fatty acid amid hydrolase (FAAH) and the monoacylglycerol lipase (MAGL) in heart and lung was performed using a Quantitect SYBR Green RT-PCR kit (DPR420A,Takara, Japan) (See Supplementary material online, Methods).

**Statistical analysis**

The SPSS 16.0 (Chicago, USA) software was used for analysis. The unpaired and paired t-tests were used for comparisons between two groups and between different conditions within the same group, respectively. One-way ANOVA with post hoc analysis by the Tukey–Kramer exact probability test was employed for multiple comparisons. Survival analysis was performed using Kaplan Meier curves with Log-rank test for comparison between the groups. Results were expressed as the mean ± SEM and P < 0.05 was considered to indicate statistical significance. All the tests were two-sided.

**Results**

**Confirmation of acute heart failure model**

One hour after TAC, left ventricular systolic pressure (LVSP) was increased to ~170 mmHg in both ICR and C57 mice (Figure 1B). Left ventricular end-diastolic pressure (LVEDP) was increased by more than 2 folds, dp/dt min was dramatically reduced, and the exponential time constant of relaxation (tau) was markedly extended (Figure 1B–E), indicating a diastolic AHF was successfully induced by TAC. Lung weight/body weight ratio (LW/BW) was significantly increased by ~90% in both ICR and C57 TAC mice (Figure 1F), indicating an acute pulmonary oedema occurred.

**Higher mortality of type one cannabinoid receptor deficiency mice in response to transverse aortic constriction**

The survival curve showed that nearly 65% of CB1 KO mice vs. 11% of WT mice died during the first week after TAC. The survival curve showed that nearly 65% of CB1 KO mice vs. 11% of WT mice died during the first week after TAC. One-way ANOVA with post hoc analysis by the Tukey–Kramer exact probability test was employed for multiple comparisons. Survival analysis was performed using Kaplan Meier curves with Log-rank test for comparison between the groups. Results were expressed as the mean ± SEM and P < 0.05 was considered to indicate statistical significance. All the tests were two-sided.

**Figure 2** Effect of type one cannabinoid receptor deficiency on 1-week survival and cause of death in response to transverse aortic constriction. (A) Kaplan–Meier survival curves for mice subjected to transverse aortic constriction. N = 35 and n = 65 in the type one cannabinoid receptor wild-type and type one cannabinoid receptor knockout groups, respectively (P < 0.001, Log-rank test). (B) Representative picture of lung with pulmonary oedema and haemorrhage. Scale bar = 3 mm. (C) The lung weight/body weight ratio for both the wild-type (n = 4) and knockout (n = 42) mice died of acute heart failure.
CB1 and acute heart failure

By daily observation of the mice and performing autopsy of the dead animals, we found that acute pulmonary oedema was the major cause of death, as indicated by the presence of pulmonary haemorrhage/oedema (Figure 2B). The LW/BW ratio was usually higher than 12 mg/g (more than two folds of the normal value) in mice that died of AHF. These results indicate that CB1 activation is crucial to protect the heart from acute pulmonary oedema.

**Figure 3** Effects of type one cannabinoid receptor deficiency on pulmonary oedema, cardiac function, and circulatory catecholamine in response to transverse aortic constriction. (A) Lung weight/body weight ratio at 1 h after transverse aortic constriction. n = 16, 9, 16, and 14 in the wild-type sham, wild-type transverse aortic constriction, knockout sham, and knockout transverse aortic constriction group, respectively. (B) Examples of congestive lungs from two wild-type and knockout mice with similar body weight subjected to transverse aortic constriction. Scale bar = 2 mm. (C) Left ventricular fractional shortening measured under anaesthesia at 10 min after surgery (n = 5 per group). (D) Left ventricular systolic and end-diastolic pressure. (E) Maximal left ventricular pressure change rate (data were transformed to percentage of the corresponding wild-type groups. n = 5, 4, 8, and 8 in wild-type sham, wild-type transverse aortic constriction, knockout sham, and knockout transverse aortic constriction groups, respectively for both (C) and (D)). Plasma levels of dopamine (F), epinephrine (G), and norepinephrine (H) at 1 h after transverse aortic constriction were measured. N = 5, 6, 7, and 12 in the Sham knockout, transverse aortic constriction knockout, Sham WT, and transverse aortic constriction WT group, respectively. *P < 0.05, **P < 0.01.
Acute cardiac effects of type one cannabinoid receptor inactivation or activation

When the mice were subjected to TAC and sacrificed after 1 h, we found that the CB1 KO mice developed more severe pulmonary oedema. There was a significant difference of LW/BW ratio between the KO and WT mice (nearly 40% larger in KO group than in WT group, Figure 3A and B), suggesting that CB1 plays an important role in pulmonary oedema.

We then examined echocardiograph and LV haemodynamic parameters of cardiac function. At 10 min after TAC, CB1 KO mice showed a significant decrease of LV fractional shortening (LV dimensions and wall thickness are showed in Supplementary material online, Figure S1), whereas no significant change was observed in WT mice (Figure 3C). At 30 min after TAC, the extent of pressure overload (i.e. the LVSP) was similar between CB1 KO mice and WT mice (Figure 3D), but, CB1 KO mice had a lower LV dp/dt max (reduced by 30%) and dp/dt min (reduced by 50%), a lower contractility index, and a larger tau (Figure 3E). These data indicated that CB1 inactivation was detrimental to both systolic and diastolic cardiac function in the context of pressure overload.

Type one cannabinoid receptor deficiency promotes catecholamine release in response to transverse aortic constriction

At one hour after TAC, catecholamine was elevated significantly in both KO and WT mice (Figure 3F–H), but CB1 KO mice had much higher epinephrine (152% of WT) and norepinephrine (231% of WT) levels than WT mice (Figure 3G and H). The decrease in CB1 activity dependent dopamine release may be one of the reasons for the lower plasma concentration of dopamine following TAC in CB1 KO mice than in WT mice, which was in agreement with previous studies. It was plausible that the extremely high epinephrine and norepinephrine levels in CB1 KO mice might have contributed to their high mortality.

Influence of epinephrine on left ventricular haemodynamic and pulmonary oedema

In both normal and TAC wild-type mice, LVEDP was significantly elevated after intraperitoneal injection of epinephrine at 2 mg/kg (Figure 4B). Epinephrine increased the LVSP, heart rate, and LV contractility index more markedly in normal mice than in TAC."

Figure 4  Acute effect of epinephrine on left ventricular haemodynamic and pulmonary oedema. (A) Left ventricular systolic pressure. (B) Left ventricular end-diastolic pressure. (C) Examples of left ventricular pressure recording. (D) The left ventricular contractility index. (E) Heart rate. *P < 0.05, **P < 0.01, compared with the respective baseline values (0). N = 7 in each group. Epinephrine (2 mg/kg) was intraperitoneal injected. (F) Epinephrine (2 mg/kg, i.p.) significantly increased lung weight to body weight ratio at 1 h after transverse aortic constriction (n = 16, 9, and 7 in the Sham, transverse aortic constriction, and transverse aortic constriction + epinephrine group). #P < 0.05, **P < 0.01.
mice (Figure 4A and C–E), suggesting that excessive sympathetic activation led to suppression of cardiac diastolic function. We further confirmed that epinephrine exaggerated acute pulmonary oedema in TAC mice indicated by a 25% increase of LW/BW (Figure 4F).

**Effects of type one cannabinoid receptor inactivation on haemodynamic, blood cell counts and endocannabinoids system**

Genotyping results are showed in Figure 5A. Intraperitoneal injection of CB1 agonist WIN exerted no effect on systolic blood pressure in CB1 KO mice while it decreased blood pressure markedly in WT mice (Figure 5B), and greater suppression of heart rate was also noted in WT mice (Figure 5C), indicating a vascular dilatory effect and a negative chronotropic character of CB1 activation.

No significant differences on peripheral blood cell counts were found between WT and KO mice in either sham or TAC groups (Figure 5D). Cardiac FAAH expression level was higher in WT sham group than in KO sham group ($p < 0.05$), which was down-regulated in WT TAC group ($p < 0.05$), whereas there was no significant difference between KO sham and KO TAC groups (Figure 5E). Pulmonary FAAH expression level was a little lower but with statistical significance in WT TAC mice than in WT sham mice ($p < 0.05$) (Figure 5F). Monoacylglycerol lipase gene expression in hearts of WT TAC mice was significantly lower

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**Figure 5** Effects of type one cannabinoid receptor deficiency on haemodynamic, blood cell counts, and endocannabinoid system. (A) Genotyping results. Effect of intraperitoneal injection of CB1 agonist WIN (Win 55, 212–2, 0.2 mg/kg) on systolic blood pressure (B) and heart rate (C) in CB1 knockout and wild-type mice. *$p < 0.05$ vs. their corresponding baselines (0 min); $n = 3$ and 5 for knockout and wild-type mice, respectively. (D) Blood cell counts, WBC, white blood cell; RBC, red blood cell; PLT, platelet; $n = 4$ in each group. Real-time PCR results for fatty acid amide hydrolase and the monoacylglycerol lipase in hearts (E) and lungs (F), $n = 6$ per group. Anandamide (G) and N-oleoylethanolamine (H) contents in hearts and lungs (pg/mg), $n = 4$ per group. *$p < 0.01$, $#p < 0.05$. 

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than in KO TAC mice ($P < 0.01$) (Figure 5E), while no significant differences were found on pulmonary MAGL expression among the four groups (Figure 5F) (also see Supplementary material online, Figure S2).

We further measured endogenous cannabinoids in heart and lung samples and found that there were no significant differences on AEA and OEA contents between WT and KO sham mice. Different from WT mice, both AEA and OEA contents in lung tissues were significantly decreased in KO mice after TAC ($P < 0.05$) (Figure 5G and H).

**Type one cannabinoid receptor agonists rescue acute heart failure**

We next tested the effects of two CB1 agonists WIN and 2-AG and a selective CB1 antagonist (AM251) on acute pulmonary oedema. These drugs were administrated before the performance of TAC. One hour after TAC, we sacrificed the mice and evaluated the LW/BW ratio. Treatment with either WIN (0.2 mg/kg) or 2-AG (5 mg/kg) significantly reduced LW/BW by $\sim 30$ and $40\%$, respectively ($P < 0.05$ and 0.01), while the administration of AM251 (0.3 mg/kg) increased it by 10% (Figure 6A). We also evaluated the effect of CB1 activation on catecholamine and LV function. In WT TAC mice, WIN did not suppress cardiac systolic function (Figure 6B), but caused a significant decrease in LVEDP by $\sim 60\%$ ($P < 0.01$), an index of pulmonary congestion, and also slowed the heart rate (Figure 6C and D).

In addition to the preventive use of CB1 agonists, we further evaluated their therapeutic effect on pulmonary congestion. One hour after the TAC, WIN, 2-AG, or AM251 was given to the mice at the same dosage as above described and LW/BW was

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**Figure 6** Effect of type one cannabinoid receptor activation on pulmonary oedema and heart function. (A) WIN 55,212-2 (WIN) (0.2 mg/kg, i.p.) or 2-arachidonyl glycerol (5 mg/kg, i.p.) significantly reduced the lung weight to body weight ratio at 1 h after transverse aortic constriction. The type one cannabinoid receptor antagonist AM251 (0.3 mg/kg, i.p.) tended to increase pulmonary oedema. $n = 16, 9, 5, 6,$ and 6 in the Sham, transverse aortic constriction, transverse aortic constriction + WIN, transverse aortic constriction + 2-arachidonyl glycerol, and transverse aortic constriction + AM251 group, respectively. (B) At 10 min after transverse aortic constriction, no significant difference on left ventricular fractional shortening was found between transverse aortic constriction and WIN + transverse aortic constriction groups. (C) Effects of pretreatment with WIN on left ventricular systolic pressure and left ventricular end-diastolic pressure. (D) WIN treatment decreased the heart rate. (E) WIN treatment decreased maximal LV pressure rise rate. $^*P < 0.05$, $^{**}P < 0.01$, compared with the transverse aortic constriction group, $n = 5$ in each group.
measured 24 h later. We noted that WIN and 2-AG reduced LW/BW by \( \approx 28 \) and 30%, respectively (\( P < 0.05 \)), while the administration of AM251 increased it by 13% (\( P > 0.05 \). Supplementary material online, Figure S4).

**Effects of type one cannabinoid receptor activation on plasma catecholamine and AMP-activated protein kinase activity**

WIN reduced circulatory epinephrine by 27% and norepinephrine by 37% in wild-type TAC mice (\( P < 0.01 \), Figure 7A). In cultured cardiomyocytes, co-culture with WIN for 30–60 min significantly enhanced AMPK activity (Figure 7B), and this effect was abrogated by knockdown of CB1 (Figure 7C). Similarly, phosphorylation of AMPK was also increased in WIN-treated WT sham and TAC mice (Figure 7D and E).

**Discussion**

In this study, we found that CB1 plays a previously unrecognized role in acute pulmonary oedema resulting from LV pressure overload. Type one cannabinoid receptor KO mice with pressure overload displayed high levels of catecholamine, severe acute pulmonary oedema, and a high acute mortality rate, while administration of epinephrine to WT mice increased LVEDP and exaggerated pulmonary oedema, indicating that excessive sympathetic activation contributed to the worsening of AHF. Since inhibition of catecholamine release is a well-recognized effect of CB1 agonists\(^8,14\) and pressure overload-induced stress stimulates CB1 and acute heart failure\(^{3131}\).

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**Figure 7** Effects of type one cannabinoid receptor activation on catecholamine levels and AMP-activated protein kinase activity. (A) Effects of pretreatment with WIN on plasma levels of epinephrine, norepinephrine, and dopamine. \( N = 6, 12, 9 \) in sham, transverse aortic constriction, and WIN + transverse aortic constriction groups, respectively, \( * P < 0.01 \). (B) Effect of WIN on AMP-activated protein kinase activity in cultured neonatal rat cardiomyocytes. (C) Effect of type one cannabinoid receptor knockdown on AMP-activated protein kinase activity. AMP-activated protein kinase phosphorylation in the hearts of sham (D) and transverse aortic constriction mice (E) was also examined. Experiments in (B) and (C) were repeated for three to four times, \( # P < 0.05, * P < 0.01 \), compared with the responding control group.
sympathetic activity, it seems reasonable that high catecholamine levels were found in CB1 KO mice after TAC. Notably, the decrease in pulmonary endogenous cannabinoids following pressure overload might have also contributed to the severer pulmonary oedema in KO mice, suggesting a cardioprotection conferred by endocannabinoids.

Most patients with AHFS present with pulmonary oedema and a normal-to-high systolic blood pressure, which is similar to our murine model of AHF. Currently available vasodilators used to treat AHFS include nitroglycerin, nitroprusside, and nesiritide, which were reported to ameliorate symptoms and improve clinical status, but did not decrease the mortality rate. Our results in the present study that mortality of AHF was increased in CB1 deficient mice and pulmonary oedema was improved by CB1 agonists in WT mice would raise new hope for the treatment of acute heart failure.

Beneficial effects of cannabinoids have been observed in various critical cardiovascular diseases such as septic shock, myocardial ischaemia/reperfusion injury, or myocardial infarction. A wealth of data have shown that cardiovascular beneficial effects could be mediated by activation of either CB2 or CB1, however, deleterious actions of CB1 activation on doxorubicin-induced cardiac dysfunction have also been reported. Emerging evidence shows that selective CB1-receptor blockade with rimonabant significantly promotes reduction in weight and favourable changes in cardiometabolic risk factors, which are at least in part attributable to blocking the role of cannabinoids on appetite stimulation.

Several recent published reports from almost the same laboratories have suggested that CB1 antagonist rimonabant is protective in some non-classical forms of heart failure induced by hepatic cirrhosis and doxorubicin. It should keep in mind that those forms of heart failure are largely different from the classical types. On the other hand, similar to beta 1 blocker, CB1 agonists have negative inotropic effect, thus it is not surprising that CB1 activation reduced myocardial contractility in hepatic cirrhosis or doxorubicine-induced heart failure. Lim et al. reported that rimonabant reduced infarct size in wild-type mice, but in untreated mice, infarct size was similar between CB1 KO and wild-type groups, suggesting that rimonabant has CB1-independent cardioprotective role. Paradoxically, a recent published large clinical trial (RESCENDO) showed that rimonabant did not improve major vascular event-free survival in obesity patients with previously manifest or underlying heart failure.

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None declared.

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