Intermedin elicits a negative inotropic effect in rat papillary muscles mediated by endothelial-derived nitric oxide

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Pires AL, Pinho M, Sena CM, Seica R, Leite-Moreira AF. Intermedin elicits a negative inotropic effect in rat papillary muscles mediated by endothelial-derived nitric oxide. Am J Physiol Heart Circ Physiol 302: H1131–H1137, 2012. First published January 6, 2011; doi:10.1152/ajpheart.00877.2011.—Intermedin (IMD) is a novel vasoactive peptide from the calcitonin gene-related peptide (CGRP) superfamily. Previous studies have shown that IMD is expressed in the pituitary gland and gastrointestinal tract, but its role in cardiovascular function is not well understood. In the present study, we investigated the direct action of increasing concentrations of IMD (10⁻⁸ to 10⁻⁴M) on myocardial performance parameters in rat left ventricular (LV) papillary muscles with and without endothelial (EE) and in presence of receptor antagonists and intracellular pathways inhibitors. In LV papillary muscles with intact EE, IMD induced a concentration-dependent negative inotropic action (P<0.05; over a baseline; at an IMD concentration of 10⁻⁴M). These effects were blunted by EE removal, AM receptor antagonist (AM22–52), and CGRP receptor antagonist (CGRP8–37).

Moreover, we investigated the role of nitric oxide (NO) synthase inhibition with L-NAME in muscles with and without EE and guanylyl cyclase inhibition with {1H-[1,2,4]oxadiazole-[4,3,4]-quinazolin-1-one} not only blunted the negative inotropic action of IMD but also unmasked IMD-positive inotropic effect dependent on CGRP receptor activation. Western blot quantification of phosphorylated cardiac troponin I (P-cTnI) in IMD-treated papillary muscles revealed a significant increase in P-cTnI when compared with untreated muscles, while in L-NAME-pretreated papillary muscles IMD failed to increase P-cTnI. Finally, we found that stimulation of both EE and microvascular endothelial cells with IMD significantly increased NO production by 40±3 and 38±3%, respectively, suggesting the role of cardiac endothelial cells in NO production upon IMD stimulation. Our findings establish IMD negative inotropic effect in isolated myocardium due to NO/cGMP pathway activation with concomitant thin myofilament desensitization by increase in cTnI phosphorylation and provide a coherent explanation for the previously reported contradictory results.

neurohormones; contractile effect; nitric oxide synthase; cardiac endothelial cells; myofilament desensitization; adrenomedullin-2

OVERWHELMING DATA SUPPORT the role of neurohumoral regulation in the cardiovascular system, as illustrated by the fact that some neurohumoral agents due to their pathophysiological function in cardiac disease have become important therapeutic targets (22). In this context, numerous studies have continued to search for new neurohormones. Among the targeted peptide families is the calcitonin gene-related peptide (CGRP) family, which includes some key neurohumoral regulators with cardioprotective function, such as adrenomedullin (ADM) and CGRP (11, 18, 33). Furthermore, recent data on genome sequence revealed a new member of this superfamily with a putative role in cardiovascular regulation, named intermedin (IMD) or ADM II (25, 32). This new peptide was synthesized in a prepro-form (prepro-IMD101–147) and a shorter 40 amino-acid peptide (IMD8–47) (25). Initial analyses revealed IMD expression mainly in the pituitary gland and gastrointestinal tract, but further immunohistochemistry studies both in rodents and humans detected a wider range of expression, including the heart. In both rat and humans, evidence suggests that IMD expression is restricted to cardiomyocytes, mainly during embryonic development of the myocardium (24, 31, 36). However, there is a study (33) describing IMD expression in endothelial microvascular cells in mice. More importantly, there are several reports documenting not only increased expression of IMD in different cardiac disease models (2, 13) but also a cytoprotective role in ischemic injury (37), indicating therefore a pathophysiological and cardioprotective function for IMD similar to ADM.

Concerning IMD cardiovascular effects, evidence from in vivo and isolated perfused heart studies demonstrated a vasodilator and hypotensive action similar to that of ADM (5, 25, 32). However, the myocardial effects remain controversial, since intravenous administration of IMD1–57 promoted a positive inotropic action, while another group using IMD1–47 observed a cardiac depressive effect accompanied by cAMP level increase (24, 35). Moreover, in isolated murine cardiomyocytes exogenous administration of IMD1–47 induced a positive inotropic effect dependent on protein kinase A (PKA) and PKC activation, concomitant with intracellular calcium release (6). Similarly, other studies investigating ADM myocardial effects have already encountered distinct inotropic action depending on the animal species and type of preparation used. In isolated rat papillary muscles and atrial cardiomycocytes, ADM elicits a positive inotropic effect (15), while it has no activity in human ventricular cardiomycocytes (26), and evokes a negative response in rabbit cardiomycocytes dependent on nitric oxide (NO) synthesis and activation of a cyclic GMP-dependent mechanism (16).

Similar to other family members IMD biological action is mediated by the G-protein-coupled receptors calcitonin receptor and calcitonin receptor-like receptor (CL), with their activity and pharmacological specificity being dependent on coexpression with receptor activity-modifying proteins (RAMPs) (21). For instance, the CL/RAMP1 complex forms the main
receptor of CGRP, while CL/RAMP2 and CL/RAMP3 complexes form the AM\(_1\) and AM\(_2\) receptors central receptors of ADM biological action (21). Unlike the peptides stated above, IMD does not exhibit selectivity for any of the three receptor complexes (25).

Regarding intracellular signaling pathways mediating IMD myocardial action, little is known and most information is extrapolated from IMD vascular studies and known second messenger pathways of other CGRP family peptides. A widely held hypothesis is the existence of two main mechanisms responsible for CGRP family peptides vasodilator action, one endothelium dependent, involving NO-cGMP pathway activation, and one endothelium independent, involving cAMP increase and PKA activation (9, 17, 36). Nonetheless, the function of cardiac endothelial cells as a possible modulator of IMD myocardial action remains unexplored.

Given the conflicting results regarding the myocardial action of IMD, as well as the lack of information concerning it modulation by cardiac endothelial cells, we investigated, the direct effects of exogenous IMD on myocardial performance and explored the role of endothelial cells and potential underlying mechanisms, including receptors, second messenger pathways, and intracellular targets.

METHODS

The study was performed in isolated left ventricular (LV) papillary muscles from Wistar-Han rats (Rattus norvegicus; 200–300 g). Rats were anesthetized with intraperitoneal pentobarbital sodium (60 mg/kg). After cardiectomy, the LV was opened and the papillary muscles were dissected free from the LV wall using a dissecting bronzed clip, and the upper tendinous end was attached to an electro- trodes arranged longitudinally alongside the entire muscle. After 10 min, bathing solutions were replaced by corresponding Krebs-Ringer solution. During the next 15 min, bathroom periods of 3, 6, and 10 min, and medium was assayed for nitrite with the Griess reaction. N\(^{\text{G}}\) monomethyl-l-arginine was added to deter- mines the number of papillary muscles. Effects of increasing concentrations of IMD were analyzed by one-way repeated-measures ANOVA. Effects of a single concentration of IMD in the various experimental condition in presence of those inhibitors or after EE damaging before the addition of intermedin. At the end of experiments, the papillary muscles were immediately freeze with liquid nitrogen and kept in −80°C freezer until use for protein quantification.

**Western blot analysis.** Western blot analysis was applied to evaluate levels of total cardiac troponin I (cTnI) and phosphorylated cTnI (P-cTnI) in papillary muscle after the experimental protocol. Briefly, tissues were homogenized on ice in 1 ml RIPA lysis buffer containing PMSF (1 mM), aprotinin (10 μg/ml), leupeptin (10 μg/ml), and pepstatin (10 μg/ml) all from Sigma Chemical (St. Louis, MO) as the protease inhibitors. Tissue was then centrifuged at 14,000 g for 20 min at 4°C. The supernatants were collected, and the total protein concentration was determined. Samples containing 40 μg of protein were loaded on to a 12% SDS-PAGE gel, run, and electroblotted onto PVDF membrane. Prestained molecular weight marker proteins were used as standards for the SDS-PAGE. Ponceau staining was performed to verify the quality of the transfer and to ensure equal protein loading. Blots were blocked in 5% skimmed nonfat milk in PBS for 1 h, treated overnight with antibody against cTnI or P-cTnI, and then incubated with alkaline phosphatase secondary antibodies for 1 h. Immunoblots were developed with an ECF Western blotting detection system (Amersham Biosciences). Protein content was determined using a Bio-Rad protein assay kit.

**EE and microvascular endothelial cell culture.** Cardiac microvascular endothelial cells (MVE) and EE cells were isolated and cultured as previously described (23). Only endothelial cells after 1 wk of isolation were used for experiments, since we observed reduction in NO production after passages 1 and 2. Confluent cell cultures were serum starved for 1 h before the start of the experiments. Purity of the cell cultures has been demonstrated previously (12).

**Determination of nitrite production upon IMD stimulation.** EE and MVE plated in 12-well culture plates were exposed to IMD for periods of 3, 6, and 10 min, and medium was assayed for nitrite with the Griess reaction. N\(^{\text{G}}\)-monomethyl-l-arginine was added to determine the role of NOS. Acetylcholine (Sigma) was used as a positive control.

**Chemicals and solutions.** All chemicals were obtained from Sigma Chemical with the exception of IMD, AM\(_{22,52}\), and CGRP\(_{5-37}\), which were obtained from Bachem (Bubendorf, Switzerland). Most of the stock solutions, including IMD, were prepared in distilled water and stored as frozen aliquots at −20°C until use, with the exception of ODQ, which was dissolved in DMSO (<0.1% in the bath).

**Statistical analysis.** Values are presented as means ± SE, and \(n\) represents the number of papillary muscles. Effects of increasing concentrations of IMD were analyzed by one-way repeated-measures ANOVA. Effects of a single concentration of IMD in the various experimental conditions were analyzed by one-way ANOVA. When significant differences were detected with any of the ANOVA tests,
the Tukey test was selected to perform pair-wise multiple compar-
sions, \( P < 0.05 \) was accepted as significant.

Experiments were subjected to the Portuguese law on animal welfare and conform to the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85–23, Revised 1996), having been performed at the Faculty of Medicine of the University of Porto, which is a governmental institution granted by the Ministério da Agricultura to perform animal research in accordance with the Decreto-Lei nº129-92.

**RESULTS**

Baseline performance of rat LV papillary muscles was similar in all experimental protocols. With exception of CGRP inhibitor (CGRP\textsubscript{8–37}) and NO inhibition with \( \text{l-NAME} \) addition, which induced a significant increase in contractile parameters (AT by \( 11 \pm 7 \) and \( 21 \pm 7\% \); \( \text{dT/dt}_{\text{max}} \) by \( 19 \pm 5 \) and \( 31 \pm 5\% \), respectively), no other receptor antagonists or inhibitors significantly changed basal muscle performance.

**Effect of IMD on myocardial performance and receptors involved.** IMD \((10^{-10} \text{ to } 10^{-6} \text{ M})\) had a dose-dependent negative inotropic effect, maximum after 3 min, at which time we observed for the maximum concentration of \( 10^{-6} \text{M}\) a significant decrease (vs. baseline) of AT by \( 14 \pm 4\% \) (Fig. 1A; \( P \leq 0.01; \) and \( \text{dT/dt}_{\text{max}} \) by \( 10 \pm 4\% \); Fig. 1B; \( P \leq 0.05 \)) with no significant effects in any other parameter. The effect of IMD was further tested in the presence of receptors antagonists \( \text{AM22–52} \) and \( \text{CGRP8–37} \), both of which significantly blunted IMD negative contractile response (Fig. 1, A and B; \( P \leq 0.05 \)). These findings suggest an inhibitory function of IMD on myocardial contractility mediated by AM and CGRP receptors.

**Role of EE removal and NO synthase and guanylyl cyclase.** Selective removal of EE blunted IMD negative inotropic response (Fig. 2; \( P \leq 0.05 \)). Furthermore, \( \text{l-NAME} \) not only blunted but even reversed IMD effects, inducing a small and slower (maximum response 6 min after administration) consistent positive inotropic response (Fig. 2, A and B). In the presence of \( \text{l-NAME} \), the IMD maximum concentration induced increased in AT by \( 7 \pm 3\% \) and \( \text{dT/dt}_{\text{max}} \) by \( 9 \pm 3\% \) (Fig. 2; \( P \leq 0.05 \)).

**Second messenger pathway and receptors involved in the positive inotropic effect of IMD upon NO synthase inhibition.** Guanylyl cyclase inhibition with ODQ (10 \( \mu \text{M}\)) promoted a positive inotropic response similar to NO inhibition, characterized by a \( 12 \pm 6\% \) increase in AT and \( 23 \pm 7\% \) increase in \( \text{dT/dt}_{\text{max}} \) (Fig. 3; \( P \leq 0.05 \)). We used PKA inhibitor H89 in association to \( \text{l-NAME} \) to explore the involvement of the cAMP/PKA pathway in IMD-induced positive inotropic response upon NO inhibition (Fig. 3). In Fig. 3, it is visible that PKA inhibition inhibited IMD-positive inotropic response. These results suggest PKA involvement in the IMD-positive inotropic response in conditions of NO depletion. Use of receptor antagonists in association with \( \text{l-NAME} \) to explore the receptor responsible for this positive inotropic response revealed opposing responses in \( \text{AM22–52} \) and \( \text{CGRP8–37} \) (Fig. 4). The presence of \( \text{CGRP8–37} \) reverted the positive inotropic response of IMD + \( \text{l-NAME} \), which induced now again a negative inotropic effect, characterized by a decrease in AT by \( 11 \pm 5\% \) and \( \text{dT/dt}_{\text{max}} \) by \( 7 \pm 4\% \). On the contrary, the presence of \( \text{AM22–52} \) did not significantly alter the positive inotropic
response of IMD + l-NAME. These results suggest role of CGRP receptors in the positive inotropic response of IMD in the presence of NO inhibition.

**IMD stimulated cTnI phosphorylation dependent on NO.** Papillary muscles treated with increasing concentrations of IMD analyzed in immunobots, presented a significant increase in P-cTnI, when compared with untreated muscles, which was characterized by an increase in the ratio of P-cTnI to total cTnI (Fig. 5). IMD addition to l-NAME pretreated papillary muscles failed to promote the increase in cTnI phosphorylation observed by a smaller increased in ratio of P-cTnI to total cTnI (Fig. 5). These results suggest that IMD stimulated cTnI phosphorylation mediated by the NO pathway.

**Effect of IMD on NO production by endothelial cells.** Further experiments in which cultured EE and MVE cells were stimulated with IMD (10^{-6} M) revealed a significant increase in nitrite production by 40 ± 8% (Fig. 6; P ≤ 0.05). These results suggest an important role of endothelial cells in myocardial response to IMD through NO production.

**DISCUSSION**

This study used a combination of functional, pharmacological, biochemical, and cell culture approaches to explore the myocardial effects of IMD and their underlying mechanisms. The main findings of the present study include the negative inotropic effect of IMD in rat papillary muscles, mediated by endothelial NO/cGMP pathway upon AM and CGRP receptor activation that leads to cTnI phosphorylation. Until now, the myocardial actions of IMD were controversial, with previous studies (6, 24, 32) in the intact heart (both in vitro and in vivo) revealing contradictory results to those observed in isolated cardiomyocytes. Moreover, the receptors and intracellular signaling pathways responsible for those actions were not comprehensively characterized. In the vasculature, there is now evidence that endothelial cells modulate IMD biological actions (24). We therefore hypothesized that endocardial and microvascular endothelial cells might also influence IMD effects on cardiac function, providing an explanation for the contradictory results in previous studies. To test this hypothesis, we evaluated the myocardial effects of IMD in rat papillary muscles in the presence of a NO synthase inhibitor and upon disruption of the endocardial endothelium. Additionally, aiming to unmask the receptors and pathways involved, myocardial performance was evaluated in the presence of several inhibitors and receptor antagonists. Furthermore, biochemical evidence was obtained by quantifying cTnI phosphorylation and therefore unveiling the role of myofilament desensitization in the myocardial response to IMD. Finally, the direct role of IMD in cardiac endothelial cells was confirmed by evaluating the IMD effects on NO production from cultured cardiac endocardial and microvascular endothelial cells.

**Contractile effects of IMD.** Similar to the in vivo studies (24, 35), we observed that exogenous administration of IMD induces a negative inotropic effect in rat LV papillary muscles, characterized by a significant decrease in muscular AT and rate of tension rise. The contractile effects of IMD were completely abolished by the receptor antagonists AM22–52 and CGRP8–37 in agreement with the general idea of IMD unspecific binding to the three receptor complexes identified to date (25). On the
IMD stimulation, since selective removal of EE blunted the function of cardiac endothelial cells in NO production upon dilator effects (12). Results from the present study suggest the role of endothelial derived NO in modulating IMD vaso- 

Moreover, there is already some evidence in vascular tissue for responsible for IMD biological action, although IMD expression on the animal species, experimental preparation, and cardiac chamber. In isolated cardiomyocytes, ADM, similarly to IMD, induced a positive inotropic action (30), while in rat papillary muscles diverse contractile effects of AMD were reported (15, 29). With regard to αCGRP, a positive inotropic effect was consistently reported (1, 14, 26). Concerning the role of cardiac endothelial cells in the modulation of CGRP peptide family contractile response, previous studies (8) from our and other groups showed that the negative inotropic action of AMD is mediated by EE in rabbit papillary muscles. However, the role of these cells in IMD myocardial contractile effects was not previously assessed.

Role of cardiac endothelial cells. Cardiac endothelial cells are potential targets for IMD in the heart and therefore likely modulators of its myocardial action. In fact, the role of these cells in the paracrine modulation of myocardial contractility, namely through the release of neurohumoral agents, such as NO and endothelin-1, among others, is well documented (3, 20). Furthermore, previous immunocytochemical studies demonstrate that these cells express the CL/RAMP receptor system responsible for IMD biological action, although IMD expression is restricted to cardiomyocytes in rats and humans (31). Moreover, there is already some evidence in vascular tissue for the role of endothelial derived NO in modulating IMD vasodilator effects (12). Results from the present study suggest the function of cardiac endothelial cells in NO production upon IMD stimulation, since selective removal of EE blunted the inotropic response to IMD. Also, stimulation of primary cardiac endothelial cells culture with the highest concentration of IMD significantly increased NO production not only in endocardial but also in microvascular endothelial cells. Regarding papillary muscles, it should be taken into consideration that this experimental preparation only allows disruption of EE, making impossible to evaluate the role of other endothelial cells, such as those present in the coronary microvasculature. The paracrine function of these cardiac endothelial cells is potentially more relevant, as they are in close contact with a much larger number of cardiomyocytes than the endocardial ones (34). These results could explain the contrasting results observed as to the IMD contractile effects in vivo and in isolated cardiomyocytes, which were until now unclear. Previously, some authors suggested IMD coronary arteries vasodilator action as a possible explanation for IMD inhibitory effect in vivo. However, our results suggest that although cardiac endothelial cells do not express IMD, they are IMD targets and responsible for NO production upon IMD stimulation.

Role of NO and cGMP. Concerning the signaling pathways responsible for endothelial cells myocardial effects, NO release by these cells is a well-known modulator of myocardial inotropic state, mainly inducing a negative inotropic effect due to soluble guanylyl cyclase activation and concomitant cGMP increase (28). With this in mind, the present study assessed the involvement of this pathway by inhibiting NO synthase by L-NAME or guanylyl cyclase by ODQ. Our results showed that inhibition of NO synthase and guanylyl cyclase not only blunted IMD negative inotropic effect but even unmasked a small positive inotropic action. These results are consistent with IMD negative contractile effects being due to NO production and guanylyl cyclase activation. It is now well established that the negative inotropic action mediated by guanylyl cyclase activation results from cGMP-dependent protein kinase (PKG) activation and inhibition of cAMP-phosphodiesterase (PDE III), with concomitant Ca^{2+} myofilament responsiveness reduction by cTnl (19). Interestingly, assessment of P-cTnl levels revealed a significant increase of this form in IMD-treated papillary muscles, which was blunted by NO inhibition. Therefore, it is reasonable to propose NO/cGMP pathway as mainly responsible for IMD negative inotropic action in rat LV papillary muscles, through myofilament desensitization due to increased cTnl phosphorylation.

Fig. 5. IMD-induced cardiac troponin I phosphorylation (P-cTnl) in rat papillary muscles. Shown are the results of immunoblots analyses in lysates prepared from papillary muscles used in the dose-response experiments: treated with IMD (n = 5); without addition of IMD [control (ctrl); n = 3]; and treated with IMD and nitric oxide inhibitor (IMD + L-NAME; n = 5). A: representative experiment. B: results from densitometric analyses of P-TnI plotted against total cTnl and normalized to actin expression. *P < 0.05 vs. ctrl.

contrary, studies in isolated murine cardiomyocytes documented a positive inotropic action of IMD mediated by PKA and PKC activation (6, 24). Comparison of the CGRP family peptides contractile effects reveals diverse responses, depending on the animal species, experimental preparation, and cardiac chamber. In isolated cardiomyocytes, ADM, similarly to IMD, induced a positive inotropic action (30), while in rat papillary muscles diverse contractile effects of AMD were reported (15, 29). With regard to αCGRP, a positive inotropic effect was consistently reported (1, 14, 26). Concerning the role of cardiac endothelial cells in the modulation of CGRP peptide family contractile response, previous studies (8) from our and other groups showed that the negative inotropic action of AMD is mediated by EE in rabbit papillary muscles. However, the role of these cells in IMD myocardial contractile effects was not previously assessed.

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Regarding the positive inotropic effect of IMD observed in the presence of NO synthase or guanylyl cyclase inhibition,
this comes in accordance with the results in isolated cardiomyocytes and corroborates our initial hypotheses of cardiac endothelial involvement in IMD myocardial effects. These results might therefore explain the differences among studies in the myocardial responses to IMD. More precisely, these results suggest that the IMD negative effect previously observed in vivo (24) is due to endothelial derived NO production, leading to cTnI phosphorylation. On the other hand, the positive inotropic action observed upon NO inhibition is due to IMD direct cardiomyocyte activation of PKA pathway, as previously shown in isolated cardiomyocytes (6). Additionally, in the present study we investigated the receptors involved in IMD positive inotropic action observed upon inhibition of NO synthase. The observation that CGRP receptor inhibition reversed the positive inotropic action of IMD in conditions of NO synthase inhibition also suggests that this receptor is the responsible for this effect. Furthermore, it is interesting to note that when this receptor was inhibited in the presence of NO inhibition, IMD had a negative inotropic action, suggesting that AM receptors activation by IMD in these conditions may activate NO-independent cGMP signaling and concomitant negative inotropic response. Additional evidence supported this hypothesis, as, for example, the fact that guanylyl cyclase inhibition in the presence of NO inhibitors and CGRP receptor antagonists (Fig. 4) blunted the negative inotropic response.

Potential pathophysiological relevance. Similarly to ADM, IMD is considered an endogenous counterregulatory peptide in the heart, opposing the detrimental effects of other neurohormones in cardiac remodeling. There are several evidences of increased IMD expression in the hypertrophic rat heart (7, 13) but more importantly of its antihypertrophic effects in isolated rat cardiomyocytes upon hypertrophic stimuli (2). Furthermore, there is evidence of IMD cardioprotective action in ischemia-reperfusion injury, associated to its negative inotropic action and antioxidant properties (10, 37). Our results, demonstrating for the first time that the myocardial effects of IMD are dependent on NO production, suggest an additional potential role for IMD in the pathophysiology of endothelial dysfunction, which is present in numerous cardiovascular diseases. Moreover, concerning CGRP, a recent work (27) reported distinct contractile effects in normotensive and spontaneous hypertensive rats hearts, due to endothelial nitric synthase downregulation in spontaneous hypertensive rats. This evidence together with our results suggests an important role of NO in CGRP family peptides action.

Conclusions. In conclusion, this is the first study to demonstrate the role of cardiac endothelial cells in the myocardial effects of IMD. We observed an IMD negative inotropic effect in rat papillary muscle, mediated by endothelial NO/cGMP pathway upon AM and CGRP receptors activation that leads to cTnI phosphorylation. Moreover, it is interesting to note the reversal of IMD inotropic response by nitric synthase and guanylyl cyclase inhibition, the first apparently dependent on CGRP but not ADM receptor activation. These results suggest an important role of NO availability in modulation of IMD myocardial action.

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GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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