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ORIGINAL RESEARCH ARTICLE

Prolonged suppression of the anti-oxidant/anti-inflammatory effects of BNP post-Takotsubo syndrome

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Abstract

Aims Takotsubo syndrome (TTS) episodes are primarily initiated by 'pulse' release of catecholamines inducing neutrophil infiltration and myocardial inflammation in susceptible individuals (largely ageing women). Evidence of myocardial inflammation and associated energetic impairment persists for ≥ 3 months post-acute TTS episodes, suggesting the existence of additional 'perpetuating' mechanisms. The effects of B-type natriuretic peptide (BNP) in suppressing superoxide (O_2^-) release from neutrophils are transiently impaired in acute heart failure. We also evaluated the extent and duration of BNP-induced suppression of O_2^- release post-TTS.

Methods and results TTS patients were studied acutely (n = 34) and 3 months thereafter (n = 13) and compared with control subjects (n = 25). O_2^- generation from neutrophils, triggered by *N*-formyl-methionyl-leucyl-phenylalanine and phorbol myristate acetate, and its suppression by BNP, were measured *in vitro*. Determinants of variability in BNP effect were sought via univariate and multivariate analyses.

Relative to control subjects, in TTS patients, BNP suppression of both phorbol myristate acetate and *N*-formyl-methionyl-leucyl-phenylalanine-induced O_2^- release was impaired acutely ($P < 0.05$ for both); this did not improve over the 3-month recovery period, despite treatment with conventional anti-failure medication in 85% of patients. No significant correlates of BNP effect (other than TTS) were identified.

Conclusions (1) While TTS is associated with marked and prolonged release of BNP, there is virtually total loss of the ability of BNP to suppress neutrophil O_2^- release and its impact on tissue inflammation. (2) BNP responses do not recover for at least 3 months post-attacks, suggesting that this might contribute to perpetuation of myocardial inflammation in TTS patients.

Keywords Takotsubo syndrome; BNP; Superoxide; Neutrophils

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Introduction

Takotsubo syndrome (TTS), also called stress-induced cardiomyopathy, apical ballooning syndrome, or broken heart syndrome, usually presents with chest pain of acute origin.[1,2] TTS occurs predominantly in ageing women, and episodes of chest pain corresponding to the onset of TTS are usually associated with emotionally or physically stressful events. Irrespective of symptoms, the diagnosis of TTS tends to be made on the basis of transient segmental left ventricular (LV) wall motion abnormalities particularly involving the LV apex or mid-ventricle without corresponding obstructive coronary artery disease.[2] Although the syndrome was first recognized and reported by a Japanese group,[3] it is now accepted that TTS occurs frequently in Caucasian populations and indeed that it accounts for up to 10% of 'heart attacks' in women aged over 50 years.[4]

The pathophysiology of TTS syndrome remains incompletely elucidated at present. Among pathophysiological mechanisms that have been proposed are multivessel coronary vasospasm, abnormalities in coronary microvascular function or spasm, catecholamine-mediated myocardial 'stunning', coronary emboli with spontaneous fibrinolysis, and/or transient obstruction of the LV outflow tract.[2,5] However, more recently, it has emerged that TTS is primarily an inflammatory 'myocarditis', with cardiac MRI investigations revealing extensive, although mainly apical, oedematous reactions.[6]

The cause of inflammation in TTS is at present not well delineated. However, excessive release of catecholamines[7] has been suggested to induce redox stress via release of reactive oxygen species and to promote inflammatory processes, resulting in myocyte dysfunction and apoptosis. Apart from cardiac magnetic resonance imaging scanning,[8] inflammation has been documented directly via endomyocardial biopsies.[7,9,10] Experimentally, a British group has provided evidence that TTS may be triggered by β_2-adrenoceptor stimulation and Gi-based signalling but did not delineate the basis for inflammatory activation.[11] Recent evidence, predominantly from studies in rodent models of TTS, has implicated the generation of peroxynitrite[12,13] and cellular infiltration with neutrophils and macrophages[12,13] in the pathogenesis of myocardial inflammation in TTS.

A number of investigations have reported markedly elevated plasma levels of B-type natriuretic peptide (BNP)[14,15] or N terminal (NT)-proBNP[16] despite the fact that the LV filling pressure is not generally elevated in TTS.[17,18] The marked and persistent elevation of NT-proBNP/BNP levels in TTS reflects both the extent of catecholamine increase and the severity of LV systolic dysfunction.[19] Furthermore, a study conducted by Morel *et al.*[20] demonstrated that in TTS patients, inflammatory activation was directly related both to the extents of impairment of LV function and neurohormonal activation. For example, C-reactive protein (CRP) levels were inversely correlated to LV ejection fraction (LVEF) and directly to BNP levels, while plasma leukocyte counts were directly correlated to both BNP and noradrenaline levels.[20] These data extend the argument that the predominant stimulus for BNP release in TTS is inflammatory rather than myocardial distension.

We have shown that BNP inhibits superoxide (O_2^-) generation in stimulated neutrophils of healthy subjects and that this effect is attenuated during the acute stages of heart failure patients.[21] Given that NT-proBNP and BNP levels are substantially elevated in the acute stages of both HF and TTS patients as well,[19] the question arises: 'Does increased BNP release "automatically" down-regulate neutrophil response?' Therefore, the objective of the present study was to determine (i) whether BNP-induced suppression of neutrophil O_2^- release is also present during the acute stages of TTS; and (ii) what are the determinants of BNP effect in individual patients with TTS.

Methods

Study cohort

Patients with TTS were prospectively identified according to the Mayo Clinic criteria.[22] The study was approved by the institutional Ethics of Human Research Committee of The Queen Elizabeth Hospital (Adelaide, Australia), and informed consent was obtained prior to study entry.

All TTS patients underwent routine clinical assessment on admission, including ECG monitoring, and regular monitoring of heart rate, blood pressure and oxygenation, transthoracic echocardiography, and coronary angiography. Additionally, blood samples were taken for determination of NT-proBNP and hs-CRP levels. Plasma levels of the endogenous catecholamine metabolites, metanephrine, and normetanephrine were determined at the time of diagnosis as previously outlined.[19]

Control subjects (without known heart diseases) were selected via advertisement and had blood samples taken subject to informed consent.

All acute TTS patients had blood taken for neutrophil evaluation at admission post-onset of symptoms. Patients were re-approached for evaluation of BNP response in neutrophils at a median of 3 months post-onset of symptoms.

Blood sampling and preparation of neutrophils

Blood samples were drawn by venesection from an antecubital vein. Blood was collected into heparinized vacutainer tubes for plasma collection or into 24 mmol/L EDTA for neutrophil preparation as described previously.[21] For platelet aggregation studies, blood was collected into plastic tubes containing 1:10 volume of citrate anticoagulant (two parts of 0.1 M citric acid and three parts of 0.1 M trisodium citrate, pH 5).

Electron paramagnetic resonance spectroscopy measurement of reactive oxygen species

For O_2^- determination by electron paramagnetic resonance (EPR), neutrophils were incubated with BNP (1 μmol/L) for 10 min and then stimulated with either PMA (100 nmol/L, for 20 min) or fMLP (1 μmol/L). Samples were scanned immediately after supplementation of the spin probe 1-hydroxy-3-methoxycarbonyl-2,2,5,5 tetramethyl pyrrolidine hydrochloride (CM-H, 200 μmol/L). EPR settings were the same as previously described.[21] EPR experiments were performed in triplicates.

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Assessment of platelet response to nitric oxide (NO) donor sodium nitroprusside

Given that platelet responsiveness to the anti-aggregatory effects of nitric oxide (NO) is impaired under oxidative stress,[23] we sought to compare integrity of platelet NO and neutrophil BNP responses in both control subjects and TTS patients. The NO donor sodium nitroprusside (SNP, 10 μmol/L) was utilized to quantitate platelet responsiveness to NO, expressed as percent inhibition of ADP-induced (2.5 μmol/L) platelet aggregation in whole blood, as previously described.[24]

Chemicals

BNP was purchased from BACHEM (Bubendorf, Switzerland). Stock solution was prepared with deoxygenated Milli-Q water, aliquoted and stored at −80°C. CM-H was purchased from NOXYGEN (Elzach, Germany). Stock solutions of CM-H (400 mmol/L) were prepared in DMSO and kept at −20°C. Working solution of CM-H (2 mmol/L) was prepared daily in Krebs-HEPES buffer. PMA, fMLP, and all other reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA). Appropriate vehicle controls were performed. Specifically, physiological effects of reagents reconstituted in DMSO (PMA) or ethanol (fMLP), and further diluted with HBSS to required concentrations, were assessed in parallel with samples subjected to analogously diluted DMSO or ethanol solutions. With reagent reconstituted in water (BNP), relevant volumes of water were added to control samples.

Data analysis

All normally distributed data are expressed as means ± standard error of the mean. Statistical significance was determined by Student's *t*-test for paired normally distributed data. GraphPad Prism version 7 for Windows (GraphPad Software, San Diego, CA) was used. Values of $P < 0.05$ were considered statistically significant.

Comparisons were made between TTS patients (n = 34) and control subjects (n = 25, aged > 40 years). Impact of increasing age was compared using Pearson's correlation coefficient and two-way ANOVA. The relationship between SNP responsiveness in platelets (a measure of integrity of soluble guanylate cyclase function) and BNP suppression of PMA-related O_2^- release (reflecting particulate guanylate cyclase function) was evaluated via linear regression for both TTS patients and control subjects. In order to evaluate the time course of putative changes in BNP response in TTS, we correlated duration of symptoms (from the time of onset of symptoms till blood sampling) with BNP response. Also, correlations were sought between BNP response and the following parameters: plasma NT-proBNP levels, peak troponin T concentrations, LVEF, lowest systolic blood pressure on admission, normetanephrine release, and utilization of angiotensin converting enzyme inhibitors, utilizing multivariate analyses.

Results

Patient characteristics

The clinical characteristics of the 34 acute TTS patients and 25 controls are summarized in *Table 1*. All patients were aged > 40 years, and none had evidence of pulmonary congestion, although LV systolic function varied substantially. One control subject had uncomplicated diabetes mellitus.

Mean age of the TTS cohort was significantly greater than that of the control subjects (mean ages, 70 ± 2 and 60 ± 2 years, respectively; $P < 0.01$). Furthermore, only 69% of the control subjects were females. Patients diagnosed with TTS were routinely treated with intravenous heparin infusion for at least 24 h.

Evaluation occurred 0.5 to 5.5 days post-onset of symptoms: this corresponded to marked but variable evaluation of NT-proBNP and hs-CRP concentrations, with 10 of the 34 patients already receiving angiotensin converting enzyme inhibitors at the time of blood sampling.

Table 1 Clinical characteristics of Takotsubo syndrome patients at admission vs. controls

	TTS patients (N = 34)	Control (N = 25)
Age (years ± SEM)	72 ± 2	60 ± 2*
Sex (M:F)	1:33	10:15*
Previous diabetes mellitus	9:25	1:24*
Minimal systolic BP (mmHg)	97 ± 3	**N/A**
Peak troponin T (ng/L)	458 ± 59	**N/A**
LVEF (%)	44 ± 2	**N/A**
Normetanephrine (pmol/L, median)	1205	**N/A**
Metanephrine (pmol/L, median)	235	**N/A**
Peak NT-proBNP (pg/mL, median)	4832	**N/A**
hs-CRP (mg/L, median)	12	**N/A**
Current ACE inhibitor therapy (n/%) (n/%)	10/29.4	**0**

ACE: angiotensin converting enzyme; BP: blood pressure; F: female; hs-CRP: high sensitivity C-reactive protein; LVEF: left ventricular ejection fraction; M: male; SEM: standard error of the mean; TTS: takotsubo syndrome.
*$P < 0.001$

Effects of BNP on neutrophil O_2^- generation in Takotsubo syndrome patients

Baseline neutrophil O_2^- content did not vary significantly between control subjects and acute TTS patients; neither did O_2^- release stimulated with PMA or fMLP significantly differ between control and patients. With regard to the impact of BNP in suppressing PMA-induced and fMLP-induced O_2^- release in TTS patients, significant attenuation was observed compared with control subjects (*Figure 1*): in general, there was no suppression of O_2^- release in the TTS population. Mean suppression values for each stimulant were, (i) for PMA: 16.2 ± 3.6% for controls vs. 0.03 ± 3.2% for TTS patients (P = 0.002); and (ii) for fMLP: 18.1 ± 5.7% for controls vs. −3.4 ± 7.8% for TTS patients (P = 0.046). In controls, BNP effects were gender independent (data not shown). In patients with TTS, BNP suppression tended to increase with patients' age (r = 0.37, P = 0.03). This trend was absent in controls but the age: BNP interaction did not vary significantly according to disease state (*Figure 2*; ANOVA: F = 1.06, P = 0.435 for interaction).

Correlations of clinical parameters with BNP effects

Among TTS patients, no univariate correlations were observed between BNP responses and normetanephrine or metanephrine concentrations, minimal systolic blood pressure, hs-CRP, LVEF, troponin T, and peak NT-proBNP levels. Furthermore, duration of symptoms (2.5 ± 0.3 days) also was not a significant univariate determinant of BNP response. If normetanephrine concentrations, minimal systolic blood pressure, LVEF, troponin T, peak NT-proBNP levels, and utilization of angiotensin converting enzyme inhibitors were forced into backwards stepwise multiple logistic regression model, none of these represented an independent correlate of BNP response among TTS patients (data not shown).

Comparison with nitric oxide (NO) signalling

In order to determine whether mechanisms and extent of BNP resistance in individual patients overlapped with those of platelet NO resistance,[21,23] correlations between these two parameters were sought. In control subjects (*Figure 3*), BNP suppression of neutrophil O_2^- release varied directly with anti-aggregation responses to SNP. This trend was blunted in TTS patients (NS: data not shown).

Impact of treatment of Takotsubo syndrome

Acute TTS patients, 13 were re-evaluated after at least 3 months' recovery. Simultaneous NT-proBNP concentration was 192 pg/mL (median, P = 0.002 vs. acute phase but still elevated above normal concentrations). BNP suppression of O_2^- release was still impaired (*Figure 4*): 1.3 ± 5.2% acutely vs. 2.2 ± 3.0% on follow-up for PMA stimulation (P = 0.9); and 2.9 ± 14.0% acutely vs. 14.4 ± 7.8% on follow-up for fMLP stimulation (P = 0.5).

Figure 1 Effect of BNP (1 uM) on neutrophil O_2^- generation in response to (A) PMA (P = 0.002) and (B) fMLP (P = 0.046) in control subjects and Takotsubo syndrome patients during index admission. Both PMA-related and fMLP-related data in controls were gender independent.

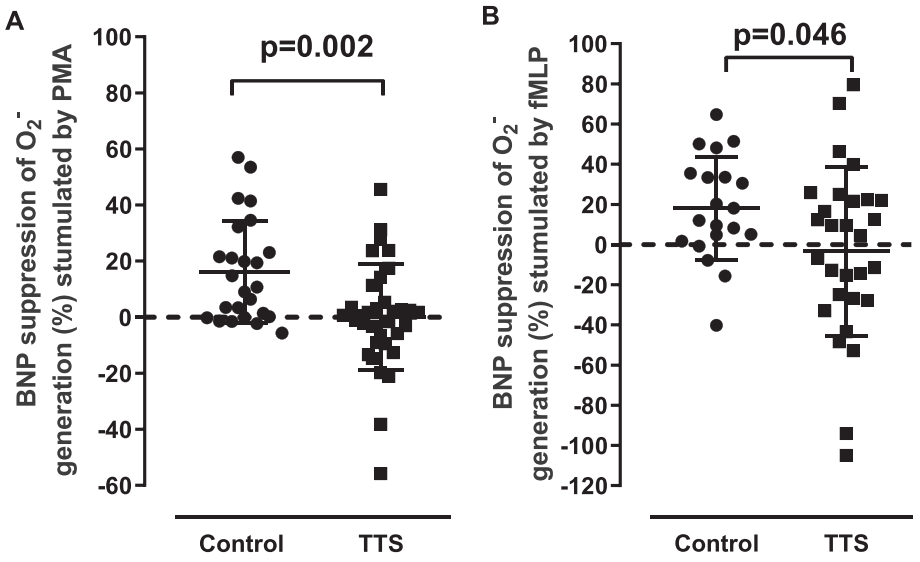

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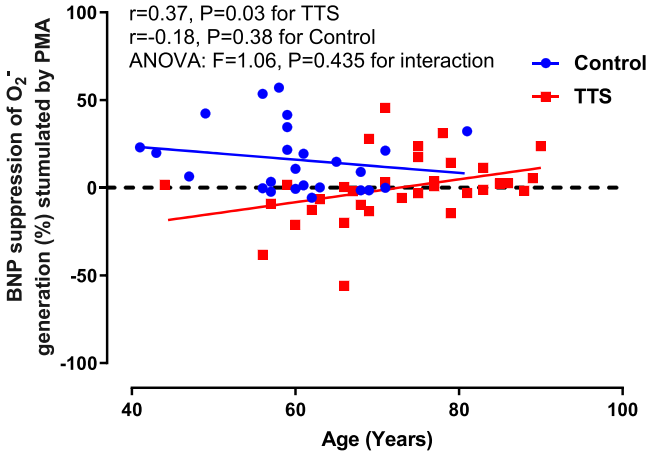
Figure 2 Correlation between age and extent of BNP effects on neutrophil O_2^- generation in response to PMA. Two-way ANOVA: $F = 1.06$, $P = 0.435$ for interaction.



Discussion

We have previously obtained data suggesting that BNP is a physiologically relevant suppressant of neutrophil O_2^- release.[25] As such, BNP might play a role in limiting the potential for extensive extracellular redox stress induced by neutrophil NADPH oxidase activation. However, these effects are substantially attenuated in patients with acute heart failure,[21] raising the possibility that this 'BNP resistance' might contribute to the extent of acute tissue (including myocardial) injury under those circumstances. The duration of BNP resistance was $<$ 3 months in that study, although it remained unclear whether the efflux of time or the initiation of treatment has restored BNP responses. Interestingly, in this heart failure cohort, NT-proBNP concentrations fell minimally with time, suggesting that plasma BNP concentrations were not the primary modulators of neutrophil BNP responses.

TTS can also be viewed as a 'heart failure syndrome',[26] although it usually presents as pseudo-acute myocardial infarction. BNP release is initially very considerable and may be triggered primarily by the intense underlying myocardial inflammation[12] rather than regional stretch. Furthermore, many TTS patients develop severe hypotension within 24 h of onset of symptoms,[27] and a role of BNP in this process has never been excluded. Hence, it is of at least theoretical interest whether the BNP released in TTS retains its physiological activities.

In the current study, we tested the hypothesis that the anti-inflammatory effects of BNP are intact in TTS. Actually, such effects measured in neutrophils were virtually

Figure 3 Correlations between BNP effects in isolated neutrophils and platelet response to sodium nitroprusside measured in whole blood of control subjects. The 19 control subjects evaluated in this way were chosen irrespective of age. $r = 0.456$, $P = 0.0496$.

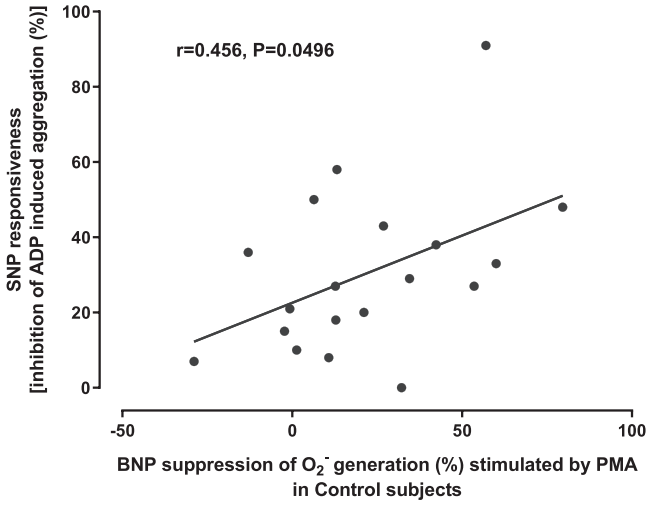


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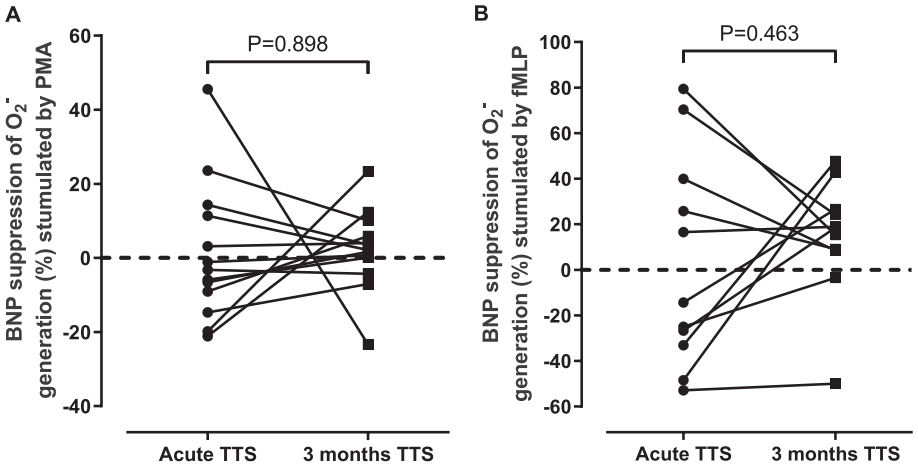
Figure 4 Comparison of BNP effects on neutrophil O_2^- generation acutely and at follow up in Takotsubo syndrome patients. (A) BNP effects on PMA-induced O_2^- generation: n = 13, P = 0.898. (B) BNP effects on fMLP-induced O_2^- generation: n = 11, P = 0.463.



abolished, both acutely and after approximately 3 months follow-up. Thus, TTS patients differ from those with acute heart failure, with prolonged suppression of BNP signalling. Notably, attenuation of BNP effects became less obvious as TTS patients aged.

It is important that both causes and consequences of the currently observed impairment of BNP responsiveness in neutrophils be considered. It must be stated that the precise cause has not been identified at this stage. We have previously explored the signal transduction pathway whereby BNP normally limits O_2^- release within the neutrophil burst,[28] and it follows from this that diminished cGMP release in response to BNP ('BNP resistance') implies a restoration of NAD(P)H oxidase assembly and resultant O_2^- release. The resultant oxidative stress may contribute to the generator of peroxynitrite, which appears pivotal to inflammatory activation in TTS.[12,29] Irrespective of mechanisms, the immediate consequence of impaired BNP effect, and consequent failure to suppress inflammation, may include aggravation of myocardial inflammation, which is prominent in both clinically based studies[30,31] and animal models of TTS.[12,13]

In TTS, neutrophil infiltration of the myocardium occurs particularly during the first few days and thereafter is much less prominent.[13] Thus, it is uncertain whether the continuation of neutrophil resistance to BNP carries the same impact on myocardial inflammatory state after the first few days. Nevertheless, it is likely that BNP resistance (which can theoretically occur within myocardium as well as neutrophils) contributes to ongoing inflammation[12] post-TTS. In theory, persistent impairment of BNP effect in patients with TTS might contribute towards slow resolution of myocardial inflammation and therefore predispose towards slow recovery of LV systolic function. Unfortunately, too few patients in the current series had simultaneous follow-up echocardiography and determination of BNP effects in neutrophils for a correlation analysis to be performed.

It should be noted that the current experiments have not evaluated the potential vasoconstrictor implications of BNP resistance in TTS or the possibility that it serves to limit hypotensive crises in the early stages post-onset of TTS. Furthermore, accentuation of BNP responses in control subjects with advancing age remains unexplained; on the other hand, the current data are clear-cut evidence that suppression of neutrophil BNP response is not purely a consequence of prolonged accentuated BNP release.

In conclusion, the current studies show that TTS represent a condition of longstanding resistance to the anti-inflammatory effects of BNP. Further studies will be needed to determine whether this concept of 'vulnerable neutrophils' should be coupled with the potential of a 'catecholamine pulse' in activating inflammation, as the overall initiating stimulus in TTS.

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Conflict of interest

None of the authors has any conflict of interest regarding the material in this manuscript.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Data S1. Supporting Info Item

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