Sex-dependent differential activation of NLRP3 and AIM2 inflammasomes in SLE macrophages

Chin-An Yang¹, Shin-Tsung Huang¹ and Bor-Luen Chiang¹,²

Abstract

Objective. SLE is more prevalent in females, but may cause more severe organ damage in males. The underlying mechanism is incompletely understood. Since macrophage plays a key role in SLE pathogenesis, the present work aimed to investigate whether inflammasomes in male and female SLE macrophages are differentially activated.

Methods. Macrophages were derived from peripheral blood mononuclear cells of SLE patients and healthy controls. Adenosine triphosphate-stimulated IL-1β in lipopolysaccharide-primed macrophages was measured via ELISA. Nucleotide-binding oligomerization domain (NOD)-like receptor pyrin domain-containing protein 3 (NLRP3) and absent in melanoma 2 (AIM2) mRNA expression in macrophages were determined by RT-PCR. We further genotyped SLE patients for single nucleotide polymorphisms of NLRP3 and an NLRP3 regulator caspase recruitment domain family, member 8 (CARD8).

Results. ATP-induced IL-1β production was increased in macrophages of both male and female SLE patients. Overexpression of NLRP3 mRNA was detected in unstimulated female SLE macrophages, while CARD8 variant allele is associated with SLE susceptibility in males. Moreover, AIM2 mRNA expression in unstimulated macrophages was found to be elevated in male SLE patients, but decreased in female SLE patients. However, the autoantibody titre of dsDNA, an AIM2 ligand, is associated with SLE disease severity only in female patients.

Conclusion. Our study shows for the first time that the NLRP3 inflammasome is hyperactivated in macrophages of both male and female SLE patients. The mechanisms underlying NLRP3 hyperactivation might be different between the sexes. Furthermore, the AIM2 inflammasome might also contribute sex-differentially to SLE pathogenesis and severity.

Key words: systemic lupus erythematosus, sex, macrophage, inflammasome.

Introduction

Marked sexual disparity, with female:male ratio of 7–9:1 has been observed in SLE [1]. Interestingly, although males are less likely to have SLE, male SLE patients often have more severe disease presentation and organ involvement [2]. The clinical observation suggests that the underlying mechanisms of SLE might be different between males and females.

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Aberrant macrophage activation and production of autoantibodies against self-nucleic acids are hallmarks of SLE. It has been suggested that oestrogen could drive autoreactive B cell proliferation and stimulate M2 macrophages [1, 2]. A single nucleotide polymorphism (SNP) in IFN regulatory factor 5 (IRF5), one major non-HLA risk allele for SLE, has been shown to have a higher frequency in males [3]. IRF5 was recently demonstrated to enhance M1 macrophage differentiation [4]. Therefore it is possible that macrophages are differentially regulated in male and female SLE patients.

Inflammasomes are multiprotein complexes containing nucleotide-binding oligomerization domain (NOD)-like receptors or absent in melanoma 2 (AIM2), which activate caspase-1 upon stimulation and promote IL-1β production [5]. Dysregulation of NOD-like receptor (NLR) pyrin domain-containing protein 3 (NLRP3) inflammasome has...
been reported in several autoimmune/autoinflammatory diseases [6, 7]. Furthermore, the AIM2 inflammasome was found to mediate apoptotic DNA-induced macrophage activation in mice with LN [8], a major disease complication in male SLE. In addition, AIM2 expression level has been shown to be increased after androgen stimulation in mice [9]. Therefore we proposed that differential activation of NLRP3 and AIM2 inflammasomes in macrophages might contribute to the distinct pathogenesis between male and female SLE.

Materials and methods

Subjects

For macrophage inflammasome stimulation assays, we recruited 15 SLE patients [6 male, age 23.2 years (s.d. 9.4); 9 female, age 23.4 years (s.d. 4.9)] diagnosed and treated at the National Taiwan University Hospital Pediatric Rheumatology Clinic. Blood samples were taken at the same time. All patients were stable at disease follow-up and had received control treatment of HCQ and steroid. The control group comprised nine male [age 28.1 years (s.d. 5.4)] and seven female [age 27.6 years (s.d. 2.3)] healthy volunteers. None of the female subjects were pregnant at the time of blood withdrawal. Sera were also collected from these subjects. For the genotyping experiment, we increased the number of subjects to 22 male SLE patients [age 24.4 years (s.d. 9.5)], 105 female SLE patients [age 26.8 years (s.d. 6.9)], 150 male age-matched controls [age 24.9 years (s.d. 5.7)] and 150 female age-matched controls [age 23.0 years (s.d. 5.3)]. Furthermore, we were able to trace the initial medical records of 13 male and 16 female SLE patients to analyse the correlation of disease severity score and anti-dsDNA titre at disease onset. All subjects are ethnic Taiwan Han Chinese. The study was approved by the National Taiwan University Hospital Research Ethics Committee. Informed consent was obtained from parents or directly from individuals if they were > 15 years old, according to the Declaration of Helsinki (October 2008).

Macrophage culture and stimulation

Human peripheral blood mononuclear cells (PBMCs) were isolated via Ficoll Paque gradient centrifugation and seeded onto 24-well plates at a concentration of $3 \times 10^6$ cells/ml in culture medium (RPMI medium with 10% heat-inactivated autoserum, 100 U/ml penicillin and 2 mM L-glutamine (all from Sigma, St Louis, MO, USA). After overnight monocyte attachment in a 37°C incubator, the non-adherent cells were removed with half of the culture medium and replaced with fresh medium each day for 7 days. On day 8, monocyte-derived macrophages were first primed with lipopolysaccharide (LPS) 1 μg/ml (Sigma) for 3 h, then stimulated with adenosine triphosphate (ATP) 2 mM (Sigma) for another 30 min, or left untreated. The culture supernatant was collected for further IL-1β analysis. Macrophages were collected and lysed with TRIzol reagent for total RNA isolation.

ELISA

IL-1β in culture supernatant was analysed by ELISA using a commercial human IL-1β ELISA kit (R&D Systems, Minneapolis, MN, USA). The serum neopterin level was detected via a human neopterin ELISA kit (IBL International, Hamburg, Germany).

RNA isolation and quantitative RT-PCR

Total RNA was extracted from $1 \times 10^6$ monocyte-derived macrophages using the RNeasy Mini kit (Qiagen). Genotyping of NLRP3 and AIM2 mRNA expression was detected by quantitative RT-PCR (qRT-PCR). The expression of beta actin (ACTB) was used as endogenous control. All primers were designed and synthesized by Genomics BioSci & Tech. The following primer sequences were used: NLRP3 (forward) GCC AAC ACT CTC GGA GAC AAG, (reverse) GCT GTG GCT GGA GTG CAG AA; AIM2: (forward) TCA GGC AGA ACC CAC AAC GT, (reverse) CCA CCT CAG CTT CCT; ACTB (forward) GTA TGG AGA AGG AGA TCA GTG C, (reverse) CAC ATC TGC TGG AAG GTG GAC. The qRT-PCR was performed on a 384-well plate using the ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA). All reactions were conducted in triplicate and the $2^{-\Delta\Delta Ct}$ method using ACTB as the calibrator was applied to calculate gene expression levels. In some experiments, primers for the nuclear localization leucine-rich repeat protein 1 (NLRP1), NLR family caspase recruitment domain family, member (CARD) domain containing 4 (NLRC4) and Mediterranean fever (MEFV) genes were also synthesized for detection of mRNA levels.

SNP genotyping

Genomic DNA was extracted from whole blood using the QiAamp Blood Mini kit (Qiagen). Genotyping of NLRP3/ OR2B11 (rs4353135) and caspase recruitment domain family, member 8 (CARD8, rs2043211) SNPs was performed by using 50–100 ng genomic DNA and fluorescence-labelled hybridization fluorescence resonance energy transfer (FRET) probes, followed by melting curve analysis on a LightCycler (Roche Diagnostics, Mannheim, Germany) as previously described [10]. The primers and hybridization probes were purchased from TIB MOLBIOL (Berlin, Germany). Primers used for NLRP3 (rs4353135) forward and reverse were GTT TCT TTT CAG AGC CTA AAC TGG and TTG CTG AGA TAT TAA GGC AAC ATC A, respectively. Primers used for CARD8 (rs2043211) forward and reverse were AGC TAC CTT TCT GTT AGA CC and ATT CAT TCT CCC TCT AGT TCG, respectively. Probes for melting curve analysis were NLRP3 sensor: GCC GCA TAC ATT TAC CCC TCFL; NLRP3 anchor: Red640-TTC TCT TTG CTT CCT TCA TTC TCT CAT TCT T; CARD8 sensor: AGC ACG GAT CAA TAA TGG CTC-FL; CARD8 anchor: Red640-CCT CTG TCT CAT CAT CTG GGA AAA AAT GT.
Statistical analysis

For comparing ATP-induced macrophage IL-1β responses between SLE patients and sex-matched controls, the Mann-Whitney U test was performed. The same test was used to analyse the difference in NLRP3/AIM2 mRNA expression level between two groups. Fisher’s exact test was applied to compare the frequency of NLRP3/CARD8 variant allele between SLE patients and sex-matched controls. A non-parametric Spearman’s test was performed to analyse the correlation between the titre of serum anti-dsDNA autoantibody and the SLEDAI score in each sex group. All statistical tests were performed using GraphPad Prism version 5 (GraphPad Software, La Jolla, CA, USA).

Results

Activation of NLRP3 inflammasome in SLE macrophages

We first examined ATP-induced NLRP3 inflammasome activation in PBMC-derived macrophages of SLE patients and sex-matched controls. LPS-primed macrophages derived from both male and female SLE patients produced higher amounts of IL-1β than the respective controls in response to ATP stimulation (male SLE median vs male control median, 391.5 vs 43.6 pg/ml, \( P < 0.01 \); female SLE median vs female control median, 353.5 vs 67.2 pg/ml, \( P < 0.01 \); Fig. 1A). Of note, female SLE macrophages stimulated by ATP alone also showed a trend towards increased IL-1β production (Fig. 1A).

Second, the NLRP3 mRNA expression level was detected by RT-PCR of RNA isolated from unstimulated macrophages. Resting female SLE macrophages showed enhanced NLRP3 expression compared with female controls (female SLE median level vs female control median level, 3.5 vs 0.7, \( P < 0.05 \); Fig. 1C), while the NLRP3 expression level was similar between macrophages of male SLE patients and male controls (Fig. 1B).

Association of NLRP3 and CARD8 variant alleles with susceptibility to SLE

In order to understand if functional SNPs in NLRP3 inflammasome components contribute to the observed macrophage hyperactivation in SLE, we further genotyped 22 male and 105 female SLE patients for rs4353135 (NLRP3) and rs2043211 (CARD8) SNPs. Remarkably, CARD8 variant A allele is associated with SLE susceptibility in males, but not in females [A allele frequency in male SLE patients vs male controls, 63.6% vs 45.3%, odds ratio (OR) 2.11 (95% CI 1.10, 4.06), \( P = 0.017 \); Table 1]. The frequency of the rs4353135 SNP was similar between each group (data not shown).

Sex-differential expression of AIM2 and correlation of serum anti-dsDNA titre with SLE disease activity

For the survey of AIM2 inflammasome activation status, we investigated the level of AIM2 mRNA in unstimulated PBMC-derived macrophages. Interestingly, AIM2 expression was significantly higher in male SLE macrophages compared with male controls (median AIM2 level, 7.2 vs 0.4, \( P < 0.01 \); Fig. 2A). In contrast, female SLE macrophages expressed significantly lower amounts of AIM2 mRNA than female controls (median AIM2 level, 0.9 vs 0.0, \( P < 0.001 \); Fig. 2B).

Furthermore, since dsDNA could not only stimulate the AIM2 inflammasome, but could also elicit B cell autoantibody production in SLE, we analysed the correlation of serum anti-dsDNA antibody titre and SLE disease severity between males and females. Of note, the serum anti-dsDNA titre correlated with the SLEDAI score in female SLE patients (\( r^2 = 0.47, P = 0.001 \); Fig. 2D) but not in male SLE patients (\( r^2 = 0.09, P > 0.05 \); Fig. 2C).

Levels of macrophage NLRP3/AIM2 expression and serum neopterin in male and female controls

To further understand if there is a sexual difference in inflammasome expression in control subjects, we compared the mRNA levels of NLRP3 and AIM2 in unstimulated macrophages of healthy males and females. While no difference in NLRP3 expression was detected (Fig. 3A), a significantly increased AIM2 level was found in female macrophages (female median level vs male median level, 634.7 vs 0.42, \( P < 0.01 \); Fig. 3B). Similarly, the mRNA expression of other inflammasome components, including NLRP1, NLRC4 and MEFV, were elevated in macrophages of control females as compared with males (Fig. 3C–E).

Moreover, neopterin is a marker of in vivo macrophage activation [11]. Consistent with previous reports [11], serum neopterin levels detected in both male and female SLE patients were higher than in sex-matched controls (pooled SLE serum neopterin median vs pooled control median, 12.2 vs 6.0 nmol/l, \( P = 0.0001 \); Fig. 3F). Of note, the neopterin level in female control serum was also elevated as compared with male controls (median nepterin in females vs males, 7.6 vs 5.1 nmol/l, \( P = 0.02 \); Fig. 3F).

Discussion

Our results showed that ATP-induced IL-1β production in LPS-primed macrophages is higher in all SLE patients. Unlike monocytes, previous priming to increase pro-IL1β and NLRP3 expression is known to be required for ATP-stimulated NLRP3 activation in macrophages [12]. The observation that ATP alone could induce IL-1β secretion in macrophages of most female SLE patients suggests the possibility of in vivo priming. Indeed, elevated levels of NLRP3 mRNA were already detected in unstimulated macrophages of female SLE patients. However, resting male SLE macrophages were found to have similar amounts of NLRP3 mRNA as male controls, which hints at a different pathway of NLRP3 activation between male and female SLE.

ATP activates NLRP3 inflammasomes by mediating K+ efflux [13]. It was recently found that self-dsDNA in the presence of anti-dsDNA antibodies can induce IL-1β production in human monocytes by activating the NLRP3 inflammasome through reactive oxygen species (ROS).
generation and K⁺ efflux [14]. Since dsDNA and anti-
dsDNA antibodies are hallmarks in SLE, NLRP3 inflamma-
somes in SLE macrophages could also be primed by this
combination in vivo. Of note, Shin et al. [14] pointed out
that dsDNA must be facilitated by its autoantibody to ac-
tivate NLRP3 inflammasomes. Oestrogen has been
demonstrated to enhance the production of IgG anti-
dsDNA in PBMCs of SLE patients [15]. Therefore it

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**Table 1** Association of CARD8 variant A allele with susceptibility to male or female SLE

<table>
<thead>
<tr>
<th>Genotype, n (%)</th>
<th>Male control 150</th>
<th>Male SLE 22</th>
<th>Female control 150</th>
<th>Female SLE 105</th>
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<tr>
<td>T/T</td>
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<td>2 (9.1)</td>
<td>50 (33.3)</td>
<td>32 (30.5)</td>
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<tr>
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<td>12 (54.5)</td>
<td>67 (44.7)</td>
<td>49 (46.6)</td>
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<tr>
<td>A/A</td>
<td>32 (21.3)</td>
<td>8 (36.4)</td>
<td>33 (22.0)</td>
<td>24 (22.8)</td>
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</tbody>
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<tr>
<th>Variant allele A, n (%)</th>
<th>Odds ratio (95% CI)</th>
<th>P-value*</th>
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<tr>
<td>Male control 150</td>
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<tr>
<td>T/T</td>
<td>136 (45.3)</td>
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<tr>
<td>T/A</td>
<td>28 (63.6)</td>
<td>2.11 (1.10, 4.06)</td>
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<tr>
<td>A/A</td>
<td>37 (44.3)</td>
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<tr>
<td>Male SLE 22</td>
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<tr>
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<td>Female SLE 105</td>
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<td>T/T</td>
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<tr>
<td>A/A</td>
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*P-value is calculated by Fisher’s exact test comparing frequencies of the A allele between sex-matched controls and SLE patients. CARD8: caspase recruitment domain family, member 8.
might be one of the reasons why the NLRP3 mRNA level is markedly elevated in macrophages of female, but not male, lupus patients.

It has been shown that functional SNPs in NLRP3 and CARD8 contribute to NLRP3 inflammasome hyperactivation [7]. The CARD8 C10X (rs2043211) variant has been reported to be associated with inflammatory activity in early RA [6]. Interestingly, the frequency of the CARD8 variant allele was significantly higher in male SLE patients as compared with male controls, while similar allele frequencies were detected between female SLE patients and female controls. Of note, it has also been shown that the combination of CARD8 and NLRP3 genetic polymorphisms predisposes the Swedish population to Crohn’s disease only in men [16]. How the CARD8 C10X variant facilitates NLRP3 activation in male SLE requires further investigation.

Defective apoptotic body clearance and aberrant macrophage activation have been proposed to be central in SLE pathogenesis [17]. An abundance of dsDNA could be derived from apoptotic bodies and further stimulate TLR9 in the endosome or the AIM2 inflammasome in the cytosol. In fact, apoptotic DNA alone has been shown to promote macrophage maturation via activation of the AIM2 inflammasome in a mouse model of LN [8]. Since LN is frequently a prominent feature in male SLE patients, we further hypothesized that in the absence of oestrogen-enhanced autoantibodies, dsDNA might cause hyperactivation of the AIM2 inflammasome in male SLE.

Consistent with our hypothesis, AIM2 mRNA expression level was found to be significantly increased in unstimulated macrophages of male SLE patients as compared with male controls. However, AIM2 expression was decreased in female SLE macrophages. The observation that the serum anti-dsDNA titre correlated only with SLE disease activity score in female patients further supports the idea that autoantibodies might play a more important role in female SLE. Indeed, the female sex hormone oestrogen is known to promote B cell maturation, antibody production and autoreactive T cell survival [1, 18–20]. Therefore autoantibody-mediated pathology could be the prominent feature in female SLE. Furthermore, self-dsDNA might prime the NLRP3 inflammasome in oestrogen-enhanced anti-dsDNA in female lupus patients, while dsDNA alone may cause AIM2 inflammasome hyperactivation in male SLE patients.

Previous studies on lupus mouse models have provided data suggesting the underlying mechanism of

Fig. 2 Sex-differential macrophage AIM2 mRNA expression and correlation of anti-dsDNA titre with SLEDAI

Relative AIM2 mRNA expression level of unstimulated macrophages derived from (A) male and (B) female SLE patients as compared with sex-matched controls. Mc: male controls, open circles; Mp: male SLE patients, open triangles; Fc: female controls, closed circles; Fp: female SLE patients, closed triangles. Lines represent medians. Male controls, n = 8; male SLE, n = 6; female controls, n = 5; female SLE, n = 9. **P < 0.01 calculated by Mann-Whitney U test. Correlation of serum anti-dsDNA level with SLEDAI in (C) male SLE patients (n = 13, Spearman correlation with regression plot, P > 0.05) and (D) female SLE patients (n = 16, significant Spearman correlation, P = 0.001). AIM2: absent in melanoma 2.
sex-dependent differential activation of the AIM2 inflammasome in SLE. Aim2 deficiency in female mice has been shown to increase the IFN-inducible Ifi200 mRNA level and the expression of other IFN-inducible genes, which correlate with lupus susceptibility [21]. Furthermore, oestrogen has been reported to up-regulate IRF5 expression [22], which not only mediates B cell differentiation [23], but could also increase p202 protein (encoded by Ifi200) expression [24] and further suppress AIM2 expression in lupus-susceptible mice [25]. In contrast, the male hormone dihydrotestosterone has been demonstrated to increase AIM2 mRNA and protein level in breast epithelial cells in mice [9]. These authors also showed that stimulation of splenic B cells from female lupus-susceptible mice decreased AIM2 expression, but increased the amount of Ifi202 RNA, another dsDNA sensor. These results indicate that oestrogen and androgen could exert distinct effects on AIM2 inflammasome activation.

Finally, we analysed the mRNA expression of NLRP3 and AIM2 in unstimulated macrophages derived from male and female control subjects. Unlike the report in lupus-susceptible mice and our observation in female SLE patients, increased AIM2 level was observed in female control macrophages as compared with those of healthy male subjects. Similarly, serum neopterin, a marker of macrophage, neutrophil and ROS activation, was found to be increased in female controls. These data suggest that oestrogen might play a role in the enhancement of human macrophage inflammasome activation and might possibly explain why females are more prone to autoimmune diseases. We mentioned in the previous paragraph that although female controls showed higher AIM2 expression than male controls, the female SLE macrophages expressed significantly lower.

Fig. 3 Comparison of macrophage inflammasome component mRNA expression and serum neopterin levels between male and female healthy controls

Relative (A) NLRP3, (B) AIM2, (C) NLRP1, (D) NLRC4 and (E) MEFV mRNA expression levels of unstimulated macrophages derived from male vs female controls. Mc: male controls, open circles; Fc: female controls, closed circles. Lines represent medians. **P < 0.01 calculated by Mann–Whitney U test. n.s.: not statistically significant. (F) Serum neopterin level of male/female controls (Mc/Fc) and male/female SLE patients (Mp/Fp). Lines represent medians. ***P < 0.001 using Mann–Whitney U test, comparing neopterin level between the control group (male + female) and the SLE group (male + female). AIM2: absent in melanoma 2; NLRP3: nucleotide-binding oligomerization domain (NOD)-like receptor pyrin domain-containing protein 3; NLRP1: nuclear localization leucine-rich repeat protein 1; NLRC4: NLR family caspase recruitment domain family, member (CARD) domain containing 4; MEFV: Mediterranean fever.
levels of AIM2 as compared with female controls. It has been suggested that in addition to oestrogen, environmental exposure and X chromosomes all contribute to lupus disease development [26]. Oestrogen enhanced anti-dsDNA antibodies only in female mice with a genetic defect in extracellular signal-regulated kinases [26]. It is likely that the suppressed AIM2 expression in female SLE patients is a result of interactions among susceptibility genes, IFN overproduction, autoantibodies and oestrogen. Further research is required to unravel the mechanism of decreased AIM2 level in female SLE patients.

In conclusion, our study shows for the first time that the NLRP3 inflammasome is hyperactivated in macrophages of both male and female SLE patients. Furthermore, the AIM2 inflammasome, which might be influenced by the effects of sex hormones and genetic susceptibilities, contributes sex-differentially to SLE pathogenesis and severity. Understanding the causes of aberrant NLRP3/AIM2 inflammasome activation in male and female SLE patients might explain the sexual difference in clinical presentation and shed light on future sex-tailored treatment for lupus patients.

**Rheumatology key messages**

- NLRP3 inflammasome is hyperactivated in macrophages of both male and female SLE patients.
- CARD8 rs2043211 polymorphism is associated with SLE susceptibility in males.
- AIM2 inflammasome might contribute sex-differentially to SLE pathogenesis and severity.

**Acknowledgements**

We thank Kai-Li Lo for helpful assistance in recruiting patients.

**Funding:** This research was supported by research funding of National Taiwan University Hospital (project number NTUH.102-S2201).

**Disclosure statement:** The authors have declared no conflicts of interest.

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