

Original Article:

# Immunological Evaluation of HIV-1 P24-Nef Harboring IFN- $\gamma$ as an Adjuvant in BALB/c Mice



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## Abstract

**Introduction:** Despite the improvements in antiretroviral treatments, there is no authorized HIV vaccine; therefore, designing an effectual vaccine is essential. This study was aimed at the immunological evaluation of HIV-1 p24-Nef adjuvanted with IFN- $\gamma$  in BALB/c mice with the purpose of stimulating effective immune responses.

**Materials and Methods:** Forty-eight female mice were used for immunization by p24-Nef. The mice were divided into six cohorts with eight mice in each group. Immunizations were executed three times at three-week intervals and subcutaneously for 5 $\mu$ g per mouse. A couple weeks after the last injection, humoral and cellular immune pathways were appraised in blood serum and splenocytes respectively through applying ELISA.

**Results:** The results showed that the applied regimen could elicit robust immune responses in comparison with the control. In addition, the level of total antibody production which was observed in the group containing adjuvanted antigen of interest had a significant difference with the control cohort ( $P < 0.0001$ ). Moreover, IgG2a was the uppermost isotype (Th1-biased response) in the immunized group that had p24-Nef antigen with IFN- $\gamma$  adjuvant. In spite of antibody secretion, the cellular immune response was the predominant stimulated pathway. The potency of IFN- $\gamma$  as an adjuvant for induction of a quantifiably extensive Th1 pathway was shown to be more significant given the outcomes of cytokine assay, IgGisotype, and CTL evaluation.

**Conclusion:** The results of the current study indicated that the p24-Nef antigen is able to stimulate the humoral and cellular immune responses in immunized mice, either on its own or when formulated with adjuvant. Thus, the high immune system stimulated by p24-Nef injection regimen went along with IFN- $\gamma$  adjuvant, offering a potential option for an efficient vaccine against HIV-1.

**Keywords:** BALB/c, Immunization, HIV-1, P24-Nef, Vaccine

## 1. Introduction

H

uman Immunodeficiency Virus (HIV) which leads to Acquired Immune Defi-

ciency Syndrome (AIDS) has moved on to be a crucial universal health apprehension. According to UNAIDS, about 38 million people were living with HIV at the end of 2019, with 68% of adults and 53% of children

infected with HIV receiving permanent Antiretroviral Therapy (ART) worldwide [1]. In the face of the developments in utilizing antiretroviral treatments, there is no authorized HIV preventative or therapeutic vaccines. Recent endeavors on providing a vaccine which could develop neutralizing antibodies against HIV-1 virus have been ineffectual [2]. Both humoral and cellular immune responses seem to be critical to avoid HIV-1 infection [3]. In contrast to usual vaccines, synthetic recombinant protein-based vaccines are more advanced and viable generally for such pathogenic virus with a remarkable quantity of mutation and limited possibility of culture in cell lines [4-6]. Recombinant protein vaccines are able to steer clear of various potential consternations caused by vaccines based on purified macromolecules, in particular the risk of co-purification of unwanted contaminants or the change of the toxoids to their toxigenic forms. The other elemental concern addressed by this technology is the intricacy involved in acquiring sufficient amount of purified antigenic components [4].

HIV-1 genome encodes three basic genes and six regulative/accessory proteins which execute all viral tasks [7]. Hence, it is expected that multifunctional proteins such as Nef, as a pivotal element in developing of AIDS, emerge [8-10]. Nef exerts this effect on AIDS pathogenesis over a few different activities, namely (i) straightly stimulating HIV-1 replication, (ii) boosting infected cells evasion from detection by the host immune system [8,10,11], (iii) downregulating cell exterior particles CD4 [12, 13] and MHC-I [14, 15], (iv) adjusting the limen activation state of T cells and macrophages [16, 17], and (v) changing signaling pathways and cellular activity which also play a key role in inducing virion infectivity and apoptosis [18]. Moreover, structural proteins including p24 protein derived from the Gag protein of HIV-1 play a fundamental role in viral center assembly and maturation [19, 20]. They highly conservative and thus an appropriate candidate for developing effective vaccines [21]. P24 protein Nef as well as P24 have been parts of various immunological trials and have proved effective to stimulate robust, multi-epitopic TCD4+ and TCD8+ immune responses [22, 23]. Cell-mediated immunity against regulatory proteins including Nef is possible and can eradicate virus-infected cells at an early stage of viral duplication given that cell-mediated immunity against the core protein p24 can obliterate infected cells by certain cytolysis [24]. Therefore, either of the mentioned HIV proteins are comprehensible targets for a multi-component vaccine. Previous research has indicated that the vaccine harboring both proteins of interest has the potential to develop enduring and effectual immune responses [25].

P24 and NEF have been considered in several studies although their fusion form has been investigated less. The recombinant peptide vaccine p24 (159-173aa) and Nef (102-117aa) along with complete Freund's adjuvant was applied to immunize BALB/c mice in a study by Mahdavi et al., which elicited CTL immune response, lymphocyte reproduction, and IL-10 and IFN- $\gamma$  generation in the Th1 immune pathway [26, 27]. Another study conducted by Gonzalez et al. revealed that the immunogenicity of full forms of p24-Nef by harboring cholera toxin B subunit as an adjuvant on BALB/c mice, evoking a robust IgG response [26, 28].

In this study, the recombinant peptide vaccine candidate of p24 (159-173aa) and Nef (102-117aa) along with interferon gamma adjuvant were applied on BALB/c mice to evaluate the immunogenicity of the fusion protein of interest as well as the protein/protein prime-boost to achieve a therapeutic vaccine candidate against HIV-1.

## 2. Materials and Methods

This study was an original experimental research in which the posttest-only control group design was used. All experiments were carried out in Genetics department of Tehran medical Sciences, Islamic Azad University, Tehran, Iran (2020).

### The preparation of recombinant HIV-1 P24-Nef peptide

The intended fusion protein for this study contains Nef and p24 immunogenic epitopes from HIV-1 virus. These peptides' sequences were found on PDB website as stated in the related study [27, 29]. PDB ID for NEF and p24 were 2 NEF and 4 XFX respectively, eventually the final sequence was achieved as "HSQRRQDILD-LWIYHTVEEKAFSPEVIPMFS".

The 31 amino acid fusion protein with 95% purity was synthesized by Takapouzi Corporation and used at the mentioned concentration (1  $\mu$ g/ $\mu$ l). In addition, interferon gamma adjuvant with 95% purity was purchased from PeproTech Corporation (Germany). To prepare the adjuvanted fusion peptide, recombinant HIV-1 Nef-p24 fusion protein was mixed with the adjuvant of interest, then was shaken for an hour. For every 200  $\mu$ g of interferon gamma, 2  $\mu$ g of candidate fusion protein was applied [29].

### Mice and immunization

48 BALB/c mice (female, 6-8 weeks old/ 18-19 g) were purchased from Pasteur Institute of Iran. Mice were treated in agreement with the universal animal care ethics. The mice were housed for 7 days prior to the experiments and given access to food and water and maintained in standard conditions (light/dark cycle (12 hrs/12 hrs) and 18 to 20°C temperature. They were separated into denoted groups as reported by Table 1. They were also immunized on days 0, 14, and 28 with 5 µg of recombinant peptides and 10 µg of adjuvant per mouse, injected subcutaneously. The mentioned fusion peptides emulsified in Freund's adjuvants (C/IFA; 50:50v/v, sigma) were also operated subcutaneously. Fourteen days after the last injection, blood samples were accumulated from retro-orbital from which the serum was segregated and saved at -80°C following heat-inactivation.

### Antibody detection utilizing ELISA

ELISA (the Enzyme-Linked Immunosorbent Assay) method was adopted to calculate antibody responses in the immunized mice. Consecutively, diluted sera from each mice group were appraised to arrive at suitable dilutions against the coated antigen of interest and accomplish quantifiable ELISA signals. In sum, 96-well ELISA Maxisorp plates (Nunk, Denmark) were covered by 100 µl of 3 µg/ml of p24-Nef recombinant proteins next the plates incubated for an overnight at 4°C. 100 µl of 1:1000 diluted sera of every mouse was prepended to the certain well and incubated at 37°C for 1h subsequent washing and blocking procedures. After washing the wells, 100 µl of 1:10000 dilution of total anti-mouse HRP-conjugated antibody (Sigma, USA) was prepended to each well; then the plates were incubated for further an hour. Finally, 100 µl of TMB (Tetramethylbenzidine; Sigma, Aldrich) was registered and the absorbance was

quantified at 450 nm. IgG antibody isotypes were recognized by goat anti-mouse IgG1, IgG2a and IgG2b antibodies (Sigma, USA) [30].

### Cytokine release assay

IL-4 and IFN-γ were determined by murine ELISA kits on the authority of the manufacture's protocol with few changes (Mabtech, Sweden). Two weeks past the latest immunization, the mice were killed in order to remove their spleens; then, the spleens were cracked into a cell homogenizer. Next, after washing the splenocytes with PBS and treating them with RBC lysis buffer for 10 min at 4°C, and centrifugation, 2×10<sup>6</sup> cells/ml were cultured in total RPMI-1640 (Gibco, Germany) medium counting 10% fetal bovine serum (Gibco, Germany), 100 U/ml penicillin, 100 mg/ml streptomycin (Biosera, USA) as well 2 mM L-glutamine (Biosera, USA) for 72 hrs along with 10 µg/ml of p24-Nef recombinant proteins. The mentioned assessments were executed three times for each group of the immunized mice. The leftover splenocytes were concomitantly utilized to assess Granzyme B secretion [31].

### Cytotoxic T Lymphocyte (CTL) assay

CTL assay was attended utilizing mouse Granzyme B ELISA kit (Invitrogen, USA). Subsequently, by purchasing Sp2/0 cell line (mouse myeloma) from National Cell Bank of Pasteur Institute of Iran, they were cultured in RPMI-1640 supplemented with 10% fetal bovine serum, 2 mM L- glutamine, 100 IU/ml penicillin/ streptomycin after that incubated at 37°C in 5% CO<sub>2</sub> with 95% humidity. Sp2/0 target T-Cells were implanted in triplicate into U-bottomed, 96-well plates (2×10<sup>4</sup> cells/well), incubated with p24-Nef recombinant antigen (10 µg/ml) for 24 hrs later. The splenocytes effector cells which were previously complete for cytokine analysis were added

**Table 1.** Immunization strategies in BALB/c mice

| Group             | Priming       | Booster 1     | Booster 2     |
|-------------------|---------------|---------------|---------------|
| PBS (Control 1)   | PBS           | PBS           | PBS           |
| C/IFA (Control 2) | CFA           | IFA           | IFA           |
| IFN-γ (Control 3) | IFN-γ         | IFN-γ         | IFN-γ         |
| P24-Nef           | P24-Nef       | P24-Nef       | P24-Nef       |
| P24-Nef+C/IFA     | P24-Nef+CFA   | P24-Nef+IFA   | P24-Nef+IFA   |
| P24-Nef+IFN-γ     | P24-Nef+IFN-γ | P24-Nef+IFN-γ | P24-Nef+IFN-γ |

PBS: Phosphate-Buffered Saline; C/IFA: Complete or Incomplete Freund's Adjuvants; IFN-γ: Interferon gamma

to the target T-cells at effector: target ratio of 100:1 in which extreme secretion of Granzyme B was detected. The target and effector cells were co-cultured in entire RPMI-1640 supplemented with 10% heat-inactivated FBS at 37°C and 5% CO<sub>2</sub> under humidified situation. Following by 6 hrs of incubation, for 5 min at 250×g and 4°C, the ELISA plates were centrifuged, also the supernatants were harvested. The concentration of GrB in the mentioned test groups was quantified on the authority of the manufacturer's guidelines [32].

### Statistical analysis

By applying Mann-Whitney non-parametric test/one-way ANOVA, the data were analyzed. The data were expressed as Mean±SD (Standard Deviation). In each single one of the test group, a p-value was deliberated over less than .05 to be statistically significant. Tests were completely executed in triplicate.

## 3. Results

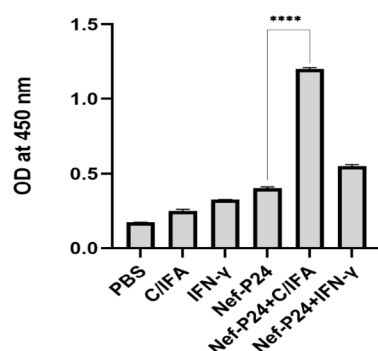
### Assessment of antibody responses

Total IgG and the IgG isotypes evaluations are indicated in (Figure 1). Each mice group immunized with fusion antigen produced HIV-1 p24-Nef-specific total IgG. Accordingly, the soaring level of total IgG was observed in the group immunized with antigen+C /IFA (P<0.0001). Besides, the level of total IgG in the mice group immunized with P24-Nef along with IFN-γ adjuvant revealed notable difference compared to control group which contained antigen alone (P<0.0001). As illustrated in Figure 2, assessment of IgG subclasses showed that IgG2a was the uppermost isotype (Th1-biased response) that was

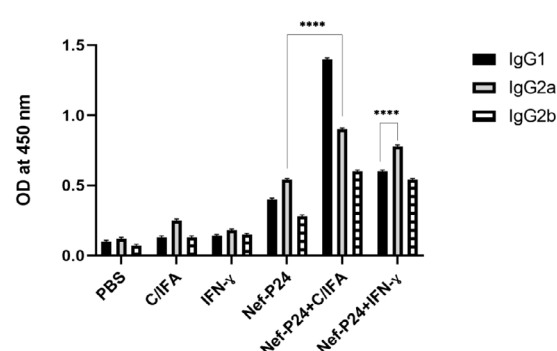
followed by IgG1 and IgG2b, respectively, revealing the remarkable difference with the level of IgG1 (Th2-biased response) in the same group (P<0.0001). Moreover, in the immunized group which adjuvanted with C/IFA, the levels of IgG2a and IgG2b were higher than pure protein recipient (P<0.0001). However, in the mentioned group the level of IgG1 significantly increased in comparison with those of IgG2 (P<0.0001). Accordingly, our data indicated that the antigen of interest when used with IFN-γ as an adjuvant resulted in higher induction of IgG2a, which revealed that Th1 pathway was engaged.

### Cytokine evaluation

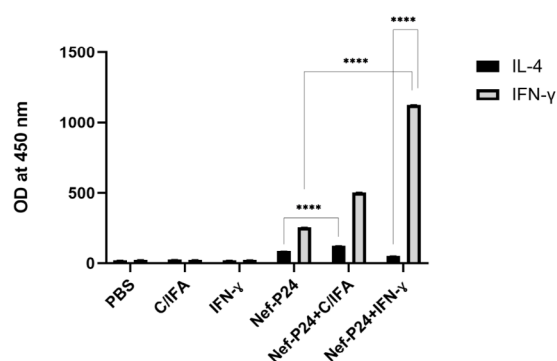
The levels of IL-4 and IFN-γ cytokines were appraised to ascertain Th2 and Th1 immune pathways in the mice splenocytes, respectively. As represented in Figure 3, IL-4 was released more in the immunized group with P24-Nef+C/IFA, which was remarkably higher than pure protein receiver group (P<0.0001). In addition, in the immunized mice group which adjuvanted with IFN-γ, the level of IL-4 was 50 pg/ml which showed a reduction (~75 pg/ml) in comparison with the level of the same cytokine in the mice group immunized with P24-Nef+C/IFA. Furthermore, the main immunized group adjuvanted with IFN-γ led to noteworthy IFN-γ generation compared to other applied formulation (1150 pg/ml). The mentioned rate in comparison with the control group (P24-Nef) revealed a significant difference in IFN-γ secretion of P<0.0001. Overall, in the groups with high IFN-γ secretion, IL-4 production decreased by contrast. Moreover, in the case of the group with the greatest cellular immune response, the rate of IL-4 release, which is a hallmark of humoral immune response, reached its lowest. On the other hand, in the same group the secre-



**Figure 1.** Analysis of humoral responses (Total IgG antibody) stimulated by various immunization strategies Utilizing ELISA. Total IgG was illustrated at 1/1000 dilution of mice sera. For each sample, all assays were done three times besides error bars which revealed ±SD in each group. \*\*\*\*P<0.0001



**Figure 2.** Analysis of humoral responses (IgG isotype-specific antibody) stimulated by various immunization strategies utilizing ELISA. Isotype specific-antibodies IgG1, IgG2a and IgG2b were illustrated at 1/2000 dilution of mice sera. For each sample, all assays were done three times besides error bars which revealed ±SD in each group. \*\*\*\*P<0.0001



**Figure 3.** IFN- $\gamma$  and IL-4 appraisal. The rates of IFN- $\gamma$  and IL-4 were assessed with sandwich ELISA as mean absorbance at 450 nm  $\pm$ SD for each set of sample. The data are presented as mean of triplicate  $\pm$  Standard Deviation (SD). \*\*\*\*P<0.0001

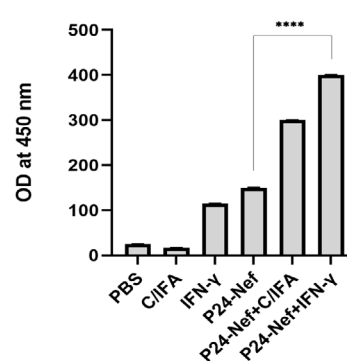
tion ratio of IFN- $\gamma$  /IL-4 was 23 pg/ml ( $P<0.0001$ ). The data indicated that in spite of antibody production, the induction rate of cellular immune response was much higher than humoral immunity.

### Granzyme B secretion

For evaluation of *in vitro* CTL activity, 3 months after the latest immunization, the secretion of Granzyme B was quantified. As observed in Figure 4, among the adjuvanted formulations, the mice group immunized with P24-Nef along with IFN- $\gamma$  adjuvant led to CTL activation remarkably compared to other groups due to the highest level of Granzyme B secretion (400 pg/ml) which showed a significant difference from the control group. According to the outcomes of cytokine assay, IgG isotype, and CTL evaluation, the potency of IFN- $\gamma$  as an adjuvant for the induction of a quantifiably extensive Th1 pathway was appraised more promising.

## 4. Discussion

The results of the current study indicated that the p24-Nef antigen is able to stimulate the humoral and cellular immune responses in immunized mice, either alone or when formulated with adjuvant. The results showed that the applied regimen could elicit robust immune responses in comparison with the control. Besides, the level of total antibody production which was observed in the group containing adjuvanted antigen of interest had a significant difference with the control cohort ( $P<0.0001$ ). Moreover, IgG2a was the uppermost isotype (Th1-biased response) in the mice immunized group that had p24-Nef antigen with IFN- $\gamma$  adjuvant. In spite of antibody secretion, the cellular immune response was the predominant stimulated pathway.



**Figure 4.** Cytotoxicity of the mice was appraised using Granzyme B (GrB) ELISA method: The results illustrate mean values worked out from triplicate samples beside the Standard Deviation (SD) as error bars. \*\*\*\*P<0.0001

Even now, detecting an influential and secure vaccine against HIV-1 is a global purpose. The use of antiretroviral combination therapy has showed success in HIV infection control although there is no eradication by now. Thus, the access to preventative or therapeutic vaccine against HIV is pivotal [33]. Failure to achieve therapeutic HIV-1 vaccine is caused by different immunological elements including inadequate post-infection natural immune response, failure in HIV replication command, utmost malformations which are foisted on the cellular immune response, and ART inability in the settled infection eradication [34, 35]. The most crucial factors which need to be considered in developing effective vaccines are the enhancement of multiple effector responses such as humoral and cellular immunity ones, antigen selection, administration procedure and delivery system [33, 34]. An effective therapeutic vaccine must be able to stimulate a wide range of immune responses, alter the host immune responses, and assist to exterminate the stockpile. The prime-boost protein/protein vaccine could be an ideal platform based on its safety and production cost. The majority of recombinant protein vaccines, if administered alone, would indicate poor immunogenicity, thus entailing the use of adjuvants to elicit a prolonged protective immune response [4, 35]. Successful utilization of recombinant proteins as vaccines containing hepatitis Band, more newly HPV, has been viable owing to the use of aluminium salt as an adjuvant [17, 36]. Thereby, the exploration of new adjuvants is a dominant field in vaccinology. The main burden for the buildout of new adjuvants includes understanding their molecular complexity and their function procedures to stimulate the immune response. Additionally, the other eminent feature that needs be considered is that an effectual formulation must be able to induce immunity against multiple epitopes by masking pivotal gene sequences; cases in point

are gag and Env as main structural genes and Nef or Vif as accessory regulation ones [37].

Therefore, with the purpose of developing a vaccine that potentially induces robust cellular and humoral immune responses, we chose P24-Nef of HIV-1 as a prospective vaccine candidate. The recombinant vaccine candidate of interest was prepared along with interferon gamma as an adjuvant to assess the cellular and humoral immune response in BALB/c mice. Nef as a regulatory peptide of HIV-1 has crucial functions in viral replication, suppression of CD4 activity, and stimulation of apoptosis in infection of the host cells [2, 38, 39]. Moreover, viral biomarkers such as HIV-1 RNA, anti-HIV antibodies, and p24 antigen have been utilized as a target antigen for early identification of HIV-1 infection [40, 41].

The results indicated that the regime of interest led to increasing stimulation of IgG2a since the C/IFA formulation activated Th2 pathway and CD4<sup>+</sup> as a result of IgG1 production. Further, IFN- $\gamma$  appraisal also revealed that the vaccine candidate led to a higher level of the mentioned cytokine induction. Moreover, CTL activity was verified in applied regimen with the greatest rate of Granzyme B secretion.

According to a study conducted in 2016 by Aghasadeghi et al., a fusion peptide of HIV-1 p24 and Nef along with the adjuvant activity of Naloxone/alum mixture was appraised in a peptide vaccine candidate. The first mice group immunized subcutaneously with the p24-Nef recombinant peptide adjuvanted with Naloxone/alum mixture and boosted with equivalent protocol. The second was immunized with fusion peptide of interest as well as alum as an adjuvant. The data showed that mice immunization with HIV-1 p24-Nef recombinant peptide adjuvanted by Naloxone/alum mixture incredibly enhanced lymphocyte proliferation and also switched cytokine responses toward Th1 pathway in comparison with the rest. In addition, HIV-1 p24-Nef fusion peptide administration while adjuvanted with Naloxone/alum mixture remarkably enlarged specific IgG responses as well as IgG1, IgG2a, IgG2b, IgG3 isotypes, and IgM vs. alum-adjuvanted vaccine regimens. Naloxone/alum mixture as an adjuvant was able to boost cellular and humoral immune responses and the mentioned adjuvant probably could be useful for HIV vaccine models in human clinical trial [42].

In another investigation, the recombinant protein of p24-Nef adjuvanted with cholera toxin B subunit was applied. Gonzalez-Rabade et al., observed a strong antigen-specific IgG answer in case of utilizing subcutane-

ous inoculation with purified chloroplast-derived p24 in contrast to Escherichia coli-derived p24. Applying oral management of chloroplast-derived p24-Nef recombinant protein as a booster thereafter subcutaneous injection with either p24 or Nef also led to a strong antigen-specific IgG response. Both IgG1 (Th1-based pathway) and IgG2a (Th2-based pathway) isotypes were observed in sera after subcutaneous and oral administrations. These data revealed that chloroplast-derived HIV-1 p24-Nef is a favorable vaccine regimen delivered through oral boosting; thus subcutaneous priming by injection of p24 and/or Nef peptides [28, 41]. What's more, in our previous study using HIV-1 p24-Nef adjuvanted with levamisole, Real-time PCR analysis indicated that IL-4 level increased remarkably ( $P < 0.05$ ) in booster groups receiving levamisole, Nef-p24, and Nef-p24/levamisole in comparison with primed cohorts. However, ELISA assay showed the rise in IL-4 expression in levamisole primed cohorts compared to Nef-p24/levamisole booster done. The data demonstrated that the Nef-p24 recombinant protein adjuvanted with levamisole could be considered effectual candidate to boost IL-4 expression which may induce the humoral immune response [29], suggesting the contribution of the equal system to HIV vaccine research.

## 5. Conclusion

The results of the current study indicated that the p24-Nef antigen is able to stimulate the humoral and cellular immune responses in immunized mice, either alone or when formulated with adjuvant. Additionally, in the evaluated formulations, the use of IFN- $\gamma$  adjuvant increased cellular immune stimulation in mice. This can pave the way for developing an effective HIV vaccine by applying other formulations and more extensive studies in other animal models.

## Ethical Considerations

### Compliance with ethical guidelines

All ethical principles were considered in this study. No human was enrolled. Animals were treated in compliance with the guidelines established by Islamic Azad University guideline based on international animal care.

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### Author's contributions

The authors equally contributed to preparing this article.

### Conflict of interest

The authors declare no conflict of interest.

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