Can transcription factors in the intervertebral disc of lopinavir/ritonavir prevent degeneration in the nucleus pulposus by mediating the regulation of inflammation through signaling pathways?

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Abbreviations

HIF-1α: Hypoxia-inducible factor-1 alpha, IVD: Intervertebral disc, IDD: Intervertebral disc degeneration, NF-kB: Nuclear factor-kappa-b, IL-1β: Interleukin-1 beta, Lop/r: Lopinavir/ritonavir, SOX9: (sex-determination region Y [SRY] protein-related high-mobility group box genes)-9.

Abstract.

OBJECTIVE: This study was conducted to examine whether lopinavir/ritonavir (Lop/r), an HIV protease inhibitor, can improve disc physiology and slow down intervertebral disc (IVD) degeneration through in vitro experimental methods, as well as whether it can suppress inflammation with interleukin-1 beta (IL-1β) and sex-determining region Y (SRY) protein-related high-mobility group box genes-9 (SOX9) through hypoxia-inducible factor 1-alpha (HIF-1α) and the nuclear factor kappa B (NF-kB) signaling pathway. The aim was to investigate whether Lop/r application is toxic to IVD cells and the microenvironment simultaneously.

PATIENTS AND METHODS: Human primary cell cultures were prepared using herniated IVD tissues obtained from patients with lumbar disc hernia who were unresponsive to conservative and medical treatment, and thereby, were operated on. The untreated culture samples served as control group, and the samples treated with Lop/r served as study group. Microscopic evaluations were performed simultaneously using fluorescent and supravital dyes in all groups. In addition to cell viability, toxicity, and proliferation analysis through a commercial kit, IL-1β, SOX9, HIF-1α, and NF-kB protein expressions were evaluated using Western blotting. In the statistical comparison of the obtained data, an alpha value less than 0.05 was considered significant.

RESULTS: Cell proliferation decreased in the Lop/r group, but no cell death was observed (p < 0.05). Moreover, at the end of 72 hours after Lop/r application, IL-1β and NF-kB protein expressions decreased by 40% and 52%, respectively, while HIF-1α and SOX9 protein expressions increased by 4% and 59%, respectively (p < 0.05).

CONCLUSIONS: Although these data were obtained from an in vitro experimental study, it is believed that these findings could make significant contributions to the pharmaco-regenerative treatment modalities of IVD degeneration. Lop/r suppresses the IL-1β and NF-kB and induces SOX9 and HIF-1α, since these signaling pathways may be related to human IVD degeneration.

Key Words: Autophagy, Disc degeneration, Hypoxia, Inflammation, Lop/r, SOX9.
Introduction

Pathologies associated with intervertebral disc (IVD) degeneration (IVDD), especially low back pain, are a global health problem that leads to high morbidity and socioeconomic burden[1,2]. To reverse or stop the degenerative process, efforts have been made to develop many different pharmacological strategies[3,4]. In addition, the regenerative effects of different pharmaceutical agents or pharmaceuticals on various modelling have recently been investigated[2,3].

In the pathogenesis of IVDD, cell death is caused by autophagy, which is the body’s way of cleaning damaged cells, and apoptosis, which is one of the main types of programmed cell death, is important to obtain healthier cells[5]. The mechanisms underlying this phenomenon include the activation of apoptotic pathways and the regulation of autophagy in response to nutrient deprivation and multiple stresses[6]. Among these pathways, proinflammatory cytokines such as interleukin (IL)-1 beta (IL-1β) or transcription factors such as hypoxia-inducible factor 1-alpha (HIF-1α), nuclear factor kappa B (NF-κB)[6], and SOX (sex-determination region Y [SRY] protein-related high-mobility group box genes)-9 play important roles[7].

Studies in mice have reported that deletion or knockout of SOX9, which has an important role in chondrocyte differentiation and prevention of IVD herniation, leads to progressive degeneration and/or progressive loss of disc cells[8]. SOX9 deletion causes IVDD characterized by apoptosis, extracellular matrix (ECM) remodeling, and compartment-specific transcriptomic changes[8]. In addition, SOX9 has been reported to regulate different signaling pathways in annulus fibrosus (AF)/nucleus pulposus (NP) cells[9-10].

Apart from the SOX9 signaling pathway, another signaling pathway that plays a critical role in the degeneration and survival of NP cells in IVD is the NF-κB signaling pathway[11]. It is known that increasing levels of IL-1β, which is one of the proinflammatory cytokines, causes IVDD to be more progressive[12]. The IVD is the largest avascular structure in the body, and IVD cells reside in vivo in an environment considered to be hypoxic[13]. HIF-1α, a heterodynamic transcription factor specific to NP, is essential in maintaining the physiological function of IVD cells[13-15].

To date[16], it has been a matter of curiosity whether many different pharmacological agents provide regeneration in many different tissues and whether they show cytotoxic effects, and such pharmaco-molecular or clinical studies have gained popularity[16-20]. Among the pharmacological agents that gained popularity is lopinavir/ritonavir (Lop/r). Lop/r and other human immunodeficiency virus type one (HIV-1) proteases were approved by the United States Food and Drug Administration (FDA) to treat HIV-1 infection[20]. In 2004, it was reported that Lop/r with a combination dose of 4 μg/ml-50 μg/ml could be effective against severe acute respiratory syndrome (SARS)-associated viruses[21]. In 2020, it was reported that anti-protease inhibitors such as Lop/r, interferons, or combinations of these drugs might become candidate drugs for SARS Coronavirus 2 (SARS-CoV-2) disease (COVID-19) treatment, SARS-CoV-2 replication, or Middle East respiratory syndrome (MERS) coronavirus replication[22,23].

Lop/r alone or Lop/r[24] has been reported to significantly increase the release of IL-1β. In a study in which Lop/r was included in the future and potential drug combinations were investigated in the treatment of SARS-CoV-2[25], the idea that abnormal host inflammatory responses could be reduced was put forward, and after the Kyoto Encyclopedia of Genes and Genomes analysis, the importance of multiple pathways, including the HIF-1α and NF-κB signaling pathways, was emphasized[26].

In this study, Lop/r, which is an anti-HIV drug, was applied to human primary AF/NP cell cultures. The data obtained after this drug administration were compared with the data obtained from the cell cultures in which drugs were not administered. On the one hand, the cytotoxic effects of Lop/r on IVD cells and ECM were evaluated. On the other hand, with Western blotting, whether the protein expressions of IL-1β, SOX9, HIF-1α, and NF-κB changed was examined. In this way, apoptosis, autophagy, and inflammation were regulated by manipulations of the aforementioned signaling pathways, and answers were sought to the questions of whether disc physiology could be improved and IVDD could be slowed down.

Patients and Methods

Case Selection Criteria

Both active substances in the Lop/r combination are inhibitors of CYP3A, an isof orm of cytochrome P450. When Lop/r was applied to the primary cell cultures to be prepared, patients who used any of the following drugs (which could interact with
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Lop/r in the last three weeks were excluded from the study for the possibility of accumulation in the tissues: alfuzosin, ranolazine, amiodarone, dronedarone, fusidic acid, neratinib, venetoclax, colchicine, astemizole, quetiapine, HMG-CoA reductase inhibitors, sildenafil, midazolam, and terfenadine. In addition, tissues belonging to pregnant women, children, and cases with renal and/or liver failure were not used in this study.

Degenerative changes in IVD were detected with the help of magnetic resonance imaging in T2-weighted sections using Pfirrmann classification. Disc tissues obtained from patients (male: 4; female: 4) who did not have modic changes and who underwent an operation due to IVD hernia, and who were stage 3-4, were used in the preparation of cell cultures. The ages of cases whose tissues (extruded or migrated disc fragments) were used varied from 26 to 40 years, with a mean case age of 32.38 ± 6.41 years.

Dissection of Tissues by Surgical Resection and Preparation of Primary Cell Cultures

Patients who were diagnosed with lumbar degenerative disc disease and whose treatment decision was to undergo surgical intervention were taken into operation following preoperative preparations. The operations of all cases were performed under general anesthesia in the prone position. Surgical intervention levels were determined with the help of fluoroscopy. Surgical areas were sterilized and covered. Midline incision was performed in all cases, and by considering the pathological level and side, lumbar facial fascia incision and subperiosteal dissections of paravertebral muscles were performed. Then, hemilaminectomy, flavectomy, and microdiscectomy were performed in appropriate patients. Following the confirmation that neural tissues were decompressed, fascia, subcutaneous, and skin sutures were made in accordance with the anatomical origin in all cases.

Degenerated IVD tissues excised during surgery were transferred to tubes containing cell culture medium and penicillin-streptomycin (PS) and transferred to the laboratory under aseptic conditions at 4°C. The tissues were broken down in petri dishes, first mechanically and then enzymatically, and taken to the incubator to be kept overnight. At the end of the period, the tissues were centrifuged at 4°C, 1200 rpm, twice consecutively for 10 minutes. The supernatant obtained was discarded. The remaining pellets were resuspended with the addition of freshly prepared cell culture medium. Samples were then transferred to individual flasks.

Cells that were viable and adhered to the surface of the flasks were counted on a Neubauer slide with Trypan blue. These cells were plated separately in well plates/petri dishes in a certain number for MTT-ELISA cell viability, toxicity, and proliferation analysis, using acridine orange/propidium iodide (AO/PI) and Janus Green-B for staining, and for Western blotting analysis in the IL-1β, SOX9, HIF-1α, and NF-κB signaling pathways to test protein expressions.

Lop/r Application to Primary Cell Cultures

In the treatment of SARS-CoV-2 infection, Lop/r is administered orally as Lop (200 mg)/r (50 mg) for 10-14 days, with two tablets in the morning and evening. Although the half-life for Lop/r is reported to be 5-6 h/3-5 h, there are also studies in the literature reporting that it is 7.4 days or 2.7 hours. In this study, considering the half-lives mentioned in the literature, the analyses were carried out from hour zero (h) to hour seventy-two.

Considering the current drug formulations, cell cultures at concentrations much higher than the achievable plasma levels are inferred, and a study in which Lop/r, darunavir, saquinavir, and atazanavir were administered concluded that "while inhibition by lopinavir was attributed to its cytotoxicity, ritonavir was the most effective of the panel, with IC50 of 13.7 µM". In cell culture studies, doses imitated to meet these doses in the clinic are reported in the literature, and in a study in which the Lop/r combination was applied to HEK-293 T cells, the IC50 dose was reported to be 10.9 µM. Therefore, in this study, the drug concentration to be applied to cell cultures was determined to be 10.9 µM.

Molecular Analyses

Morphological evaluations of cell and ECM structure were performed with the help of inverted light microscopy using different magnifications such as 4x, 10x, 20x, and 40x. Then, AO/PI staining was applied to all cell samples by applying Janus Green-B staining, which is one of the supravital stains, to examine whether there was mitochondrial and DNA damage and whether the cells were alive.

In addition, a3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was per-
formed using a commercial kit (Vybrant MTT Cell Proliferation Assay, Cat. No. V-13154; Cell Biolabs, San Diego, CA, USA), and cell viability, toxicity, and proliferation analysis were performed with the enzyme-linked immunosorbent assay (ELISA) in accordance with the commercial kit instructions.

In addition to histopathological evaluations with the help of a microscope, Western blotting was performed after sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) in Lop/r and non-Lop/r samples simultaneously. HIF-1α (cat no. MA1516), IL-1β (cat no. M421B), phosphor-NF-κB P65 (ser536, cat no. MA515181), and SOX9 (cat no. 14-9765-82) were evaluated with the help of commercial antibodies obtained from Thermo Fisher Scientific, Waltham, MA, USA. β-actin (cat no. MA511869, Thermo Fisher Scientific, Waltham, MA, USA) was used as the internal control gene (housekeeping gene, endogenous control). Total protein concentration in the protein lysates from each sample was determined spectrophotometrically with a Bradford assay.

To determine the protein expression levels by immunoblotting, proteins were first separated by the 10% SDS-PAGE method. Then, a polyvinylidene difluoride (PVDF) transfer membrane was used, which provides high binding capacity for proteins and nucleic acids and is an ideal membrane for the transfer of proteins. Immunoblotting for these target proteins was performed using the Western-breeze Chemiluminescent kit (cat no. WB7104, Thermo Fisher Scientific, Waltham, MA, USA) in accordance with the manufacturer’s instructions. Subsequently, after the treatment with the primary antibody specific to these proteins, sequential washings were performed. After incubation with alkaline phosphatase-conjugated secondary antibody, the membranes were washed three times. In the last stage, treatment with substrate solution was performed. The protein bands transferred to an X-ray film (Thermo Fisher Scientific, Cat no. 34090, Waltham, MA, USA) were analyzed using ImageJ software, where the specific amount of protein in each sample was determined.

Statistical Analysis
Whether there was a difference between the groups was evaluated by one-way analysis of variance (ANOVA), and which group was different from the other was evaluated by Tukey’s honestly significant difference test (HSD), a post-hoc test. The evaluations were carried out with the help of Minitab (version 22) with a 95% confidence interval, and \( p < 0.05 \) was considered statistically significant. The results were presented as mean ± standard deviation (StDev), frequency (%), minimum, and maximum.

Results
In the cells in the Lop/r study group, there was a decrease in proliferation rates of 86.90%, 62.36%, and 51.74% at hours 24, 48, and 72, respectively, compared with the control group of pure primary cell cultures in which no drugs were administered (Table I, Figure 1). These results were also statistically significant (\( p < 0.05 \) (Table II).

Morphological examination showed that the data obtained from MTT-ELISA were supported. However, although proliferation was suppressed, the cell morphology did not change in Lop/r samples and cell viability continued. Cell death was not observed at the doses and durations applied in AO/PI staining. In the Janus Green-B staining results, the color of the indicator, which is blue, remained, and the pink color that occurs in an oxygen-free environment was not seen (Figure 2).
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β-actin, which is the housekeeping gene, was used for normalization in the evaluation of fold changes of proteins in Lop/r samples and was accepted as 100% (1-fold), and protein expression in each group was calculated based on β-actin (Table III).

Western blotting evaluations revealed that IL-1β protein expression decreased by 2% at hour 24, increased by 13% at hour 48, and decreased by 40% at the end of the hour 72 in the Lop/r group. It was observed that NF-kB protein expression decreased by 37% (0.63-fold) at hour 24, 39% at the end of hour 48, and 52% at the end of hour 72. HIF-1α value increased by 27%, 120%, and 4% at hours 24, 48, and 72, respectively. The increase in SOX9 protein expression at the same time points was calculated as 36%, 55%, and 59%, respectively (Figure 3).

All the increased and decreased protein expressions were found to be statistically significant ($p < 0.05$).

Discussion

Understanding the molecular mechanisms regulating the maintenance and destruction of IVD could lead to the development of new therapies for IVDD\textsuperscript{35}. One of the mechanisms that play an important role in the mechanism of IVDD is autoph-

<table>
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<th>F-Value</th>
<th>$p$-value*</th>
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Table II. MTT-ELISA analysis of cell viability, toxicity, and proliferation of AF/NP cells following Lop/r treatment.

Figure 1. Interval plot of absorbance (540 nm, O.D.) of the experimental group treated with Lop/r compared with the control group. MTT analyzes were repeated at least three times.

Table III. Comparison of r values between groups obtained after Western blotting.

<table>
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<tr>
<th>Groups</th>
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<td>0.48</td>
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<td>1.59</td>
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</tbody>
</table>

* $p < 0.05$ vs. control group and $p < 0.05$ vs. Lop/r-treated group. Data were analysed using a one way analysis of variance.
SARS-CoV in *in vitro* studies. As of the 2019 pandemic, it has been frequently used in many clinics around the world, including Turkey. In a randomized controlled and open-labelled study, gastrointestinal adverse events such as diarrhea and nausea were reported to be more common in the Lop/r group compared with the groups receiving other antiretroviral drugs. In addition, a study in which it is associated with pancreatitis is also found in the literature. Information such as increases in total bilirubin, triglyceride and liver enzymes levels during Lop/r treatment, hypersensitivity reactions, Stevens-Johnson syndrome, toxic epidermal necrolysis, prolongation of QT interval, and Torsade de Pointes is also included in the literature.

Although the etiology of IVDD is not clear, excessive oxidative stress, inflammation, disruption of apoptosis, and autophagy play important roles in the pathogenesis of IVDD.

The dual effects of autophagy on cells are that an appropriate level of autophagy can protect cells, whereas excessive autophagy will cause cell death. IVDD is closely related to the loss of ECM, apoptosis, and inflammation in NP cells.

Lop/r has been shown to be effective against MERS in animal experiments in the past, and there is limited evidence that it is also effective in MERS/SARS patients. Lop/r, which is a protease inhibitor and is used in the treatment of HIV infection, has been reported to be effective on SARS-CoV in *in vitro* studies. As of the 2019 pandemic, it has been frequently used in many clinics around the world, including Turkey.

In a randomized controlled and open-labelled study, gastrointestinal adverse events such as diarrhea and nausea were reported to be more common in the Lop/r group compared with the groups receiving other antiretroviral drugs. In addition, a study in which it is associated with pancreatitis is also found in the literature. Information such as increases in total bilirubin, triglyceride and liver enzymes levels during Lop/r treatment, hypersensitivity reactions, Stevens-Johnson syndrome, toxic epidermal necrolysis, prolongation of QT interval, and Torsade de Pointes is also included in the literature.

Figure 2. Microscopic images performed at hours 0, 24, 48, and 72. A, B, and C constitute the control groups without drug administration, and the other photographs are the Lop/r samples. A, D, G, and J are images of non-stained cell classes in an inverted light microscopy (20× magnification). B, E, H, and K are the images obtained after Janus Green-B staining (20× magnification). C, F, I, and L are post-AO/PI staining images (10× magnification).
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Figure 3. Effect of Lop/r on inflammatory/degeneration-related protein expression in human IVD cells. The expression levels of the above proteins were determined by SDS-PAGE following Western blot. Western blot analyzes were repeated at least three times, protein expression levels were determined in each independent experiment with the ImageJ software and averaged.

However, the effects of Lop/r on AF/NP cells and ECM structure are not mentioned in the literature. Therefore, although this study is a first, we believe that despite it being an in vitro experimental study, the data obtained can contribute to the literature.

HIV-1 protease inhibitor ritonavir and integrase inhibitor raltegravir have been reported to increase the expression levels of many proinflammatory cytokines, especially IL-1β. Excessive apoptosis in IVD cells is important in IVDD, and IL-1β has been shown to induce apoptosis in these cells. It is known that the deterioration in proteoglycan structure, which contributes to the pathogenesis of IVDD, is induced by inflammatory cytokines such as IL-1β. Cell autophagy is involved in many diseases, especially IVDD, including osteoarthritis. Autophagy induced by IL-1β can cause different molecular mechanisms depending on the type of cell. However, the effect and mechanism of autophagy regulation by IL-1β in IVDs remain unclear. IL-1β induces apoptosis and autophagy through the mitochondria pathway in human degenerative NP cells.

Induction of autophagy also decreases intracellular levels of pro-IL-1β. In particular, autophagy modulates the transcription, processing, and secretion of IL-1β, acting as an important negative feedback mechanism for the control of inflammatory responses, both in vitro and in vivo. In this study, we observed that protein expression of IL-1β, which is a proinflammatory cytokine, decreased in AF/NP cell cultures with the addition of Lop/r.

In addition, inflammatory responses in IVDs are regulated by NF-κB. Several investigations have characterized the upregulation of inflammatory factors during IVDD progression. Of these inflammatory factors, IL-1β contributes to IVDD by upregulating catabolic enzymes, inhibiting normal ECM production, and increasing cell apoptosis.

Whereas inhibition of autophagy in rat NP cells increases the catabolic effect of cytokines, activation of autophagy has been described to suppress the catabolic effect of cytokines. In addition, autophagy of rat NP cells has been reported to be induced by NF-κB inhibition under inflammatory conditions.

This study found that the protein expression of the NF-κB signaling pathway decreased in addition to the decrease in protein expression of IL-1β, in AF/NP cell cultures with Lop/r addition.

SOX9 is known to have anabolic effects that regulate ECM. ECM degradation is induced by decreasing collagen I, collagen II, SOX9, and aggrecan expression by inhibiting autophagy. HIF-1α, on the other hand, is generally known to activate mitophagy, which is known as the elimination of damaged mitochondria and its participation in the cellular recycling process after the stressful period. HIF-1α regulates chondrogenesis by regulating SOX9 expression at the genetic level. HIF-1α is also involved in both autophagy and apoptosis regulation. Therefore, HIF-1α maintains the viability of chondrocyte cells by promoting the chondrocyte phenotype and can protect the articular cartilage by supporting metabolic adaptation to a hypoxic environment.

Janus Green-B is applied to stain the mitochondria supravitally in cells. Evaluations are made by taking advantage of the color change according to the amount of oxygen available in the organelle. When oxygen is present, the indicator is oxidized to a blue color, whereas in the absence of oxygen, the indicator decreases, and the color turns pink.

In this study, it was observed that proliferation was suppressed, but there was no cell death after Lop/r administration. In addition, when the culture samples to which Lop/r was applied were evaluated after Janus Green-B staining, it was understood that AF/NP cells did not enter hypoxia.
as a result of the blue coloration of the mitochondria. In addition, it was understood that protein expressions of SOX9 and HIF-1α transcription factors increased during the experiment in the Lop/r group.

Lop/r could play an important role in inhibiting inflammation through autophagy mediated by NF-κB in NP cells and ameliorating IVDD. The fact that the IL-1β/NF-κB signaling pathway has catabolic effects on disc tissue cells in IVDD cascade and SOX9 and HIF-1α have anabolic effects, in line with the findings obtained from this study, strengthens the idea that Lop/r can be used in the treatment of IVDD in the future.

**Limitations**

The limitations of this study include the in vitro study design and lack of in vivo validation of the observed effects of IL-1β, SOX9, HIF-1α, and NF-κB signaling pathways on IVDD. In addition, apart from the genes associated with apoptotic or autophagic cell death, which we could not evaluate within the scope of this study, the investigation of genes that are effective in cell cycle regulation is still one of the questions that need to be answered.

**Conclusions**

Because Lop/r both reduces cytokine response via IL-1β and suppresses inflammatory responses via the NF-κB signaling pathway, and on the other hand, it balances hypoxia via HIF-1α and induces SOX9, which plays an important role in the anabolic pathway, it could slow down, stop, or perhaps regenerate human IVDD. As such, Lop/r could represent a potential target for novel anti-inflammatory therapies. However, these data were obtained from in vitro experimental studies and cannot mimic the clinical aspect exactly. Therefore, there is a need for results to be obtained from more detailed in vivo experimental and clinical studies.

**Availability of Data and Materials**

The data and materials generated/analyzed in the present study are available from the corresponding author upon request.

**Consent for Publication**

Not applicable.

**Ethical Approval**

This research was carried out with the permission of Izmir Bakircay University School of Medicine, Local Ethics Committee, dated 12/01/2022 and numbered 486.466.

**Authors’ Contributions**

IY is the principal author of this study and designed the study and the experiments and provided the dissolution of the drug in suitable solvent and applied Lop/r to the cell cultures they prepared from the tissues. Worked on the experiments and the pharmacological analysis of the samples. HA: Provided the dissolution of the drug in suitable solvent and applied Lop/r to the cell cultures they prepared from the tissues and collected the data. NK: Identified the cases to be included in the study and surgically resected the tissues from the cases to be used in the preparation of AF and NP cell cultures and critical revision. HA and DYS: Worked on the experiments and the molecular analysis of the samples. NK, MD, NeK, and OA: Inclusion criteria of the cases whose tissues were used and surgical resection of tissues. IY, DYS, NK, NeK, HO, and OA: Revising the article for scientific and intellectual content; IY, MD, and HO: Statistical analysis and interpretation of the data; IY, DYS, NK, MD, and HO: Final approval of the version to be published. All authors read and approved the final manuscript.

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