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# Limiting Immunogenicity of Diabetic Retinopathy Patient-Derived Induced Pluripotent Stem Cells by Knocking out Human Leukocyte Antigen and Overexpressing Cluster of Differentiation 47

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**Abstract**

**Introduction:** Diabetic retinopathy (DR), the most common complication of diabetes, is characterized by vision loss due to vascular endothelial cell damage of the retina. Notably, induced pluripotent stem cell (iPSC) therapy has shown promise in the regeneration of the retina after damage. A limitation of using these cells includes the risk of immune-rejection. The knockout of human leukocyte antigen (HLA) proteins prevents a host immune response to non-native cells; however, HLA depletion introduces natural killer (NK) cell-mediated responses. Overexpression of cluster of differentiation 47 (CD47) inhibits the activity of NK cells. This project aims to create a universal DR patient-derived iPSC platform whose immunogenicity is limited through genetic alterations.

**Methods:** iPSCs will be reprogrammed from DR patient-derived fibroblasts and CRISPR-Cas9 will be used to knock out HLA and overexpress CD47. Alterations will be validated through Sanger sequencing, Western Blot and Immunofluorescence (IF) analysis. DR patient-derived iPSCs will be differentiated into endothelial cells to mimic the DR afflicted endothelial cells. These differentiated cells will then be co-cultured with NK cells and a cytotoxicity assay will be performed. Specifically, a chromium-release assay will be used by loading radioactive chromium into the genetically modified and unmodified endothelial cells and the chromium released by dying cells was monitored.

**Results:** Compared to the unmodified cells, we expect less NK-mediated cell death for the genetically altered endothelial cells.

**Discussion:** Effectively limiting the immunogenicity of the donor-derived iPSCs can establish a universal platform for future studies in DR therapy.

**Conclusion:** The resulting donor-derived iPSCs can be used to test drug therapies for DR or new methods to repair blood vessel damage, among a multitude of new research.

**Keywords:** diabetic retinopathy, regenerative medicine therapy, limiting immunogenicity, CRISPR-Cas9, CD47

## Introduction

Diabetic retinopathy (DR) is a disease of the eyes stemming from the long-term effects of diabetes. High blood sugar levels cause vision-threatening damage to the neurons and blood vessels that compose the light-sensitive tissue of the retina, eventually leading to blindness [1]. DR affects approximately 93 million people worldwide [2] and is the leading cause of visual impairment in younger, working-age populations [3,4,5]. Blindness is the indirect result of neovascularization in the retina, which is the process of the creation of abnormal blood vessels stimulated by a multitude of factors. These new abnormal blood vessels can lead to vitreous hemorrhage, and oftentimes tractional retinal detachment, which ultimately leads to blindness [6]. Individuals with either Type 1 or Type 2 diabetes mellitus are at risk of developing this disease and of those with diabetes mellitus, the risk of being affected by DR is greater in those who have had it for longer [4]. Exposure to high levels of glucose in the blood for a longer duration of time can cause neural and/or vascular damage and/or death which can be attributed to the extended periods of retinal toxicity [7].

The pathology of DR prevents cells from participating in the repair of the diseased or damaged retina. Initially, in response to hyperglycemia, the blood vessels of the retina dilate to accommodate the increased uptake of excess glucose from the bloodstream. Alongside this change is the loss of pericytes, which are vital to the structural integrity of capillaries. As these pericytes apoptose or become damaged, they create bulges in the walls of the capillary [5]. These bulges are called microaneurysms and are the most common initial sign of DR [8]. Beyond these

characteristic signs of DR, the presence of DR may also be indicated by endothelial cell apoptosis, and/or a thickening of the basement membrane [5]. An inflammatory response may also be initiated by the immune system in retinal neurons as a response to hyperglycemia, which is a major contributor to the alterations of the retina [9].

The treatment of DR remains a challenge as the effects of the disease cannot be reversed. Current treatments such as intravitreal pharmacologic agents, laser photocoagulation and vitreous surgery can delay progression of the disease [5]. Intravitreal administration of antivascular endothelial growth factor (anti-VEGF) agents shrink the new blood vessels formed and decrease the macular edema. Laser usage slows the leakage of fluid and blood from the abnormal blood vessels that form in the eye, whilst a vitrectomy helps to preserve vision by allowing drainage of vitreous humor, which hinders further vascular changes [5]. Notably, these treatments are invasive and fail to provide clinically-significant improvements in the vision of DR patients [5]. Stem cell based therapies offer a new approach to manage this debilitating disease.

Recent literature reviews on stem cell therapies in DR discussed the use of induced pluripotent stem cell (iPSC) therapy as a potential treatment for DR and have shown promise in the regeneration of the retina after damage [3,10]. However, the application of regenerative medicine therapy is limited due to the risk of immune rejection to the iPSCs [3,10]. In a previous study, stem cells that are "invisible" to the immune system were created via the knockout of Major Histocompatibility Complex (MHC) Class I and II and overexpression of the protein CD47. This has been seen to reduce the risk of immune rejection of iPSCs in immunocompetent mice recipients [11]. Using this technique on DR patient-derived iPSCs, a universal platform for future studies in DR therapy can be established. The aim of this protocol is to address the risk of immune rejection through the introduction of genetic alterations of the donor-derived iPSCs that will limit their immunogenicity.

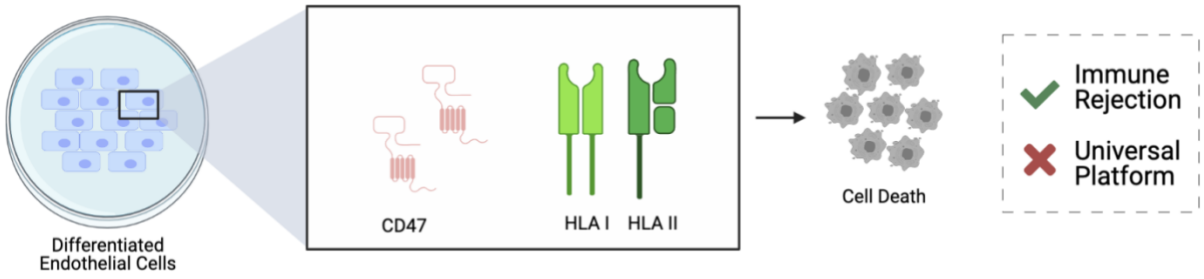


**Figure 1.** Diagram indicating the components of unmodified immunogenic iPSCs, and their resulting death when co-cultured with natural killer (NK) cells. Created with BioRender.com.

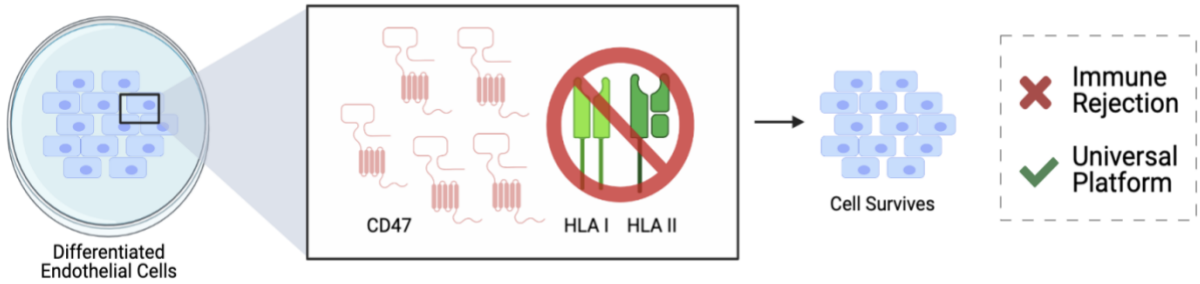


**Figure 2.** Diagram indicating the components of modified immunogenic iPSCs, and their resulting survival when co-cultured with natural killer (NK) cells. Created with BioRender.com.

Diabetic retinopathy is an incurable disease that affects the vision, thus negatively impacting the quality of life of those suffering from it [4]. Individuals and their family members' lives can be affected through a variety of aspects including emotional stress, financial loss and strain on familial relationships, education, and work [4]. Thus, taking steps toward a solution can help improve these individuals' lives.

MHC is a locus of DNA on the human chromosome 6 that codes for cell surface proteins, particularly those that are responsible for immune responses to foreign entities [3]. MHC Class I and II, known as the Human Leukocyte Antigen (or HLA) Class I and II regions in humans, are responsible for mediating cell immune responses. The knockout of MHC Class I and II genes in mice has previously been seen to prevent the host cell from attacking non-native cells. However, it was observed that the depletion of MHC can be detrimental to the cell as it introduces Natural Killer (NK) cell mediated responses [11]. NK cells are lymphocytes belonging to the innate immune system and attack foreign cells in the body [12]. The overexpression of a protein known as CD47 has shown to inhibit the phagocytic tendencies of these NK cells [11]. We hypothesize that through the strategic knockout of immunity genes in HLA Class I and HLA Class II and overexpressing CD47, the immunogenicity of DR patient-derived iPSCs can be limited to survive in the presence of NK cells. These patient-derived iPSCs will have the potential and added advantage of limited immunogenicity and are virtually invisible to the immune system. This will establish a universal platform for future studies in DR therapy.

## Methods

### Synthesis of iPSCs

To ensure the integrity and quality of our research we will attain ethical clearance for this study and agree to comply with the Tri-Council Policy Statement (TCPS2) for conducting this research with human participants. When conducting this study, we will seek informed consent from all research participants, and respect their confidentiality throughout the progression of the study.

Human fibroblast cells will be obtained from consenting individuals who have been diagnosed with DR for a minimum of 2 years and will be retrieved from both males and females aged 20 years, with the age of diagnosis being 18 years or older. DR patient-derived fibroblasts will be obtained through a superficial punch skin biopsy conducted on a DR-afflicted patient under local anesthesia [13]. Fibroblast cells will be cultured in a XF-HFFm at 37°C then reprogrammed into iPSCs via retroviral transfection encoding FLAG-tagged Oct4, Sox2, Klf4, and c-Myc which will be introduced and left in contact with the cells for 24h at 37°C [11]. After 2 rounds for infection, fibroblasts will be dissociated and placed onto iXF-HFF plates coated with CELLStart (Invitrogen) in F44m or XF-hESm. This medium will be replaced on a daily basis over a 10 day period. Following this period, one million cells will be plated onto each plate. Based on morphology, colonies will be picked a month after the initial plating onto iXF-HFF plates coated with CELLStart (Invitrogen) [14].

### β2 microglobulin (B2m) Class I HLA gene knockout

These iPSCs will undergo gene-modification steps through CRISPR-Cas9 to knock out the Beta-2 microglobulin (B2m) gene, a structural component of HLA Class I. Two single guide RNA (sgRNA) sequences complementary to the HLA I B2m target gene will be used in this knockout. This will be accomplished by ligating B2m genes into vectors with a Cas-9 expression cassette and transfecting them into iPSCs [11]. The guide protein sgRNA 1 will be used to flank the start codon of the genes for B2m, and sgRNA 2 to make a four base pair insertion [15]. This will be followed by a 14 base pair deletion to introduce new stop codons that will prematurely terminate the translation of the in the HLA I region [15]. Sanger sequencing will be used to confirm that the B2m knockout was successful.

### Class II major histocompatibility complex transactivator (CIITA) Class II HLA gene knockout

The B2m−/− iPSCs will be subsequently transfected with a vector containing CRISPR-Cas9 that will be used to target the knockout of the Class II transactivator (CIITA) which acts as a regulator for HLA Class II molecules. All-in-ONE (AIO) vectors containing a Cas9 expression cassette will be ligated with the appropriate CRISPR sequence. The iPSCs will then be transfected and dissociated using 0.05% trypsin (Gibco) [11]. After the expansion of single cells into full colonies, cells will be screened for the evidence of altered sequence at the CRISPR cleavage site using the GeneArt™ Genomic Cleavage Detection Kit (Thermo Fisher). A Polymerase Chain Reaction (PCR) kit (Guide-it™ Indel identification kit) will be used to amplify the target sequence, followed by Sanger sequencing to produce a chromatogram indicating the presence of the altered sequence at the CRISPR cleavage site. To verify the deletion of CIITA, the Guide-it Indel identification kit will be used to determine Indel size.

### Cluster of Differentiation 47 (CD47) Overexpression

A copy of the mRNA coding for CD47 will be created and cloned into a lentiviral plasmid (pLenti6/V5) that contains an antibiotic resistance cassette for blasticidin [11]. Sanger sequencing will check for mutations in the iPSCs for the absences of overexpressed CD47. B2m−/−Ciita−/− iPSCs will be grown on blasticidin-resistant MEF cells and transduced with this lentiviral plasmid for 72 hours. Antibiotic selection will occur for 7 days after blasticidin is introduced to select for blasticidin resistant cells [11]. Immunofluorescence imaging will be completed on antibiotic resistant cells using the LIVE/DEAD cell Imaging kit to confirm the overexpression of CD47 in the modified iPSCs. Western blot analysis will also be completed to confirm the overexpression of CD47.

Differentiation of iPSCs into DR-afflicted Endothelial Cells

Based on the research conducted by Shinya Yamanaka from 2006, whereby iPSC technology was studied, a handful of methods arose for differentiation of iPSCs into DR-afflicted endothelial cells [16]. Since then, numerous studies have produced effective methods for differentiation which include co-culture, embryoid body formation, two-dimensional culture with growth factors, and three-dimensional (3D) culture [10,17,1819,20]. Of these various methods, the two-dimensional culture with growth factors presents a shorter period of production time (i.e., 1 week compared to the typical 2-3 weeks production time) and greater yield of endothelial cells (about 60%) [18]. In this method, differentiation is separated into two distinct stages. In stage 1, cells will be cultured on a nutrient-rich medium to promote proliferation and differentiation into endothelial cells [18]. In stage 2, endothelial cells will rely on the extracellular matrix (ECM) for nutrients needed for survival [18]. Both stages use a combination of growth factors to differentiate and efficiently produce endothelial cells.

A chromium release cytotoxicity assay, commonly used to measure the damage done to cells by natural killer (NK) cells [11], will be a verifying method used to compare the modified, differentiated, DR-afflicted endothelial cells to unmodified, differentiated DR-afflicted endothelial cells to determine their percentage of lysis when co-cultured with NK cells.

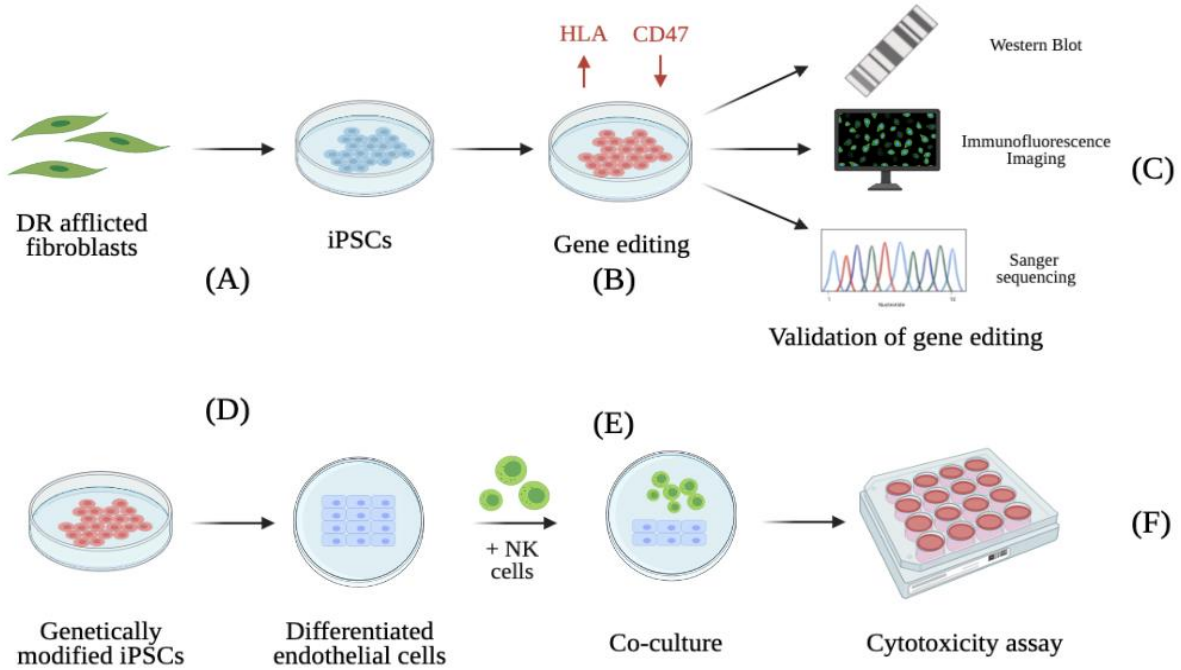


**Figure 3.** This diagram depicts the chronological methods, and processes used to procure differentiated endothelial cells from DR afflicted fibroblasts. (A) DR patient-derived fibroblasts will be collected and reprogrammed into iPSCs. (B) CRISPR-Cas9 will be used to genetically edit the HLA Class I and II regions to knock out B2m and CIITA and overexpress CD47 in the iPSC. (C) Alterations will be validated through Sanger sequencing, Western Blot and IF analysis. (D) Differentiating the iPSCs into endothelial cells to mimic the DR afflicted endothelial cells. (E) Differentiated cells will then be co-cultured with NK cells and (F) a chromium-release assay will be performed by loading radioactive chromium into the genetically modified and unmodified endothelial cells. Created with BioRender.com.

**Results**

Proposed results of this prospective study will depict the immunogenicity of modified endothelial cells developed from DR-afflicted patients. This protocol is primarily an outline for a prospective study, thereby all results will remain descriptive and non-numerical, and their analyses will be hypothetical. To obtain numerical data as verification of the genetic alterations statistical analysis will be conducted to compare levels of HLA and CD47 in the differentiated endothelial cells (previously unaltered fibroblasts) of DR-patients to the unaltered fibroblasts of the DR-afflicted individuals. The primary statistical method of use is a multivariate analysis of variance (MANOVA) with the differentiated endothelial cells and the unaltered fibroblasts of the DR-patients being the two independent variables, and the levels of HLA and CD47 being the two dependent variables. Interpretation of the results of the MANOVA with a 95% confidence interval, $P < 0.05$ will be

considered statistically significant. The purpose of this statistical method is to determine whether or not the differences between the groups of data are statistically significant. The expected results of this study should confirm differences in the levels of HLA and CD47 in DR patient-derived fibroblasts from the levels of HLA and CD47 in the differentiated endothelial cells.

**Discussion**

This research protocol aims to provide a methodology and experimental design to introduce stem-cell therapy in the treatment of DR in afflicted patients. This can be done through the creation of iPCSs that evade the immune system and can be used universally for the treatment of DR.

Proposed results will include the analysis of B2m Class I HLA gene knockout. This will be seen from the Sanger sequencing results of CRISPR-Cas9 mediated sgRNA B2m knockout sequence chromatogram that is expected to display the 4-bp insertion and 14-bp deletion. Additionally, data from a Western blot will also indicate B2m knockout and will include lanes for a control, targeting with sgRNA 1, targeting with sgRNA 1 & 2 and targeting with sgRNA2. In the control lane, and in the lanes targeting only sgRNA 1, and only sgRNA 2, a presence of B2m will be evident, whilst the lane concurrent with both sgRNA 1 and 2 targeted will indicate an absence of B2m.

Our method includes the analysis of class II major histocompatibility complex transactivator (CIITA) Class II HLA gene knockout. Expected results will display a Sanger sequencing of CRISPR-Cas9 mediated sgRNA CIITA knockout sequence chromatogram indicating a 1-bp insertion. The Western blot data for CIITA knockout will include two lanes: a control and targeting with sgRNA 1. In the control lane, the presence of CIITA is anticipated to be evident whilst the lane indicating sgRNA 1 usage should depict an absence of CIITA.

Expected results also include the analysis of the CD47 overexpression which will be depicted by Western blot data. Lanes are expected to include a control and a lane with the genetically modified cell. The control should indicate a normal presence of CD47, while the lane of the genetically modified cell should display a darkened blot in accordance with the overexpression of CD47. Additionally, an Immunofluorescent staining for CD47 in unmodified cells and genetically altered endothelial cells is also warranted to confirm the overexpression of CD47 in the DR afflicted iPSCs.

Results can be validated further through assessment of cell viability using a chromium-release cytotoxicity assay. A cytotoxicity profile of modified and unmodified DR-afflicted cells in the presence of NK cells can be analyzed. Analysis of the modified DR afflicted cells that undergo overexpression of CD47 and knockout of HLA genes is expected to exhibit a significantly lower percent lysis than the unmodified DR afflicted cells.

A hurdle in the use of iPSCs to treat DR is the risk of immune rejection [3, 21]. Previous studies have aimed to overcome barriers posed by adverse immunogenic responses in the body through gene editing. A paper authored in 2019 successfully showed the ability of both mouse and human iPSCs derived from endothelial, cardiomyocytes and smooth muscle cells to lose immunogenicity following the knockout of MHC Class I and II proteins and the overexpression of CD47 [11]. Following these findings, our proposed research aims to apply these gene edits to produce iPSCs derived from DR-afflicted patients. In doing so, these genetically edited iPSCs can be differentiated into endothelial cells to potentially treat the damaged endothelial cells of the retina in DR-patients in future studies.

Current treatments such as intravitreal pharmacologic agents, laser photocoagulation and vitreous surgery work to slow the progression of DR. However, many of these treatments have damaging side effects which pose limitations when treating DR [5]. Intravitreal administration of antivascular endothelial growth factor (anti-VEGF) agents shrink the new blood vessels formed and decrease the macular edema [5]. Despite being one of the most common treatments for DR, studies have shown that improvement in best-corrected visual acuity (BCVA) was only attained in 29% of patients using anti-VEGF therapy for two years [5, 22]. This low rate of improvement has been attributed to potential disruptions in molecular pathways of the body after exposure to anti-VEGF treatment [5, 22]. By using genetically modified iPSCs that can survive undetected in the body, disruptions in other molecular pathways posed by anti-VEGF treatments can be mitigated.

Laser photocoagulation is another common method of treating DR. Focal/grid macular laser therapy targets edema of the macula in order to improve vision in DR-patients [5, 23]. However, laser therapy is invasive and can damage retinal cells, which may lead to a loss of central vision and night vision [5, 24].

Steroids such as intravitreal corticosteroids are also used in the treatment of DR [25, 26]. These are generally not the first option for treatment as there is a high prevalence of associated adverse effects [25, 26]. Steroids tend to increase the intraocular pressure of the eye leading to glaucoma. These drugs are also associated with the risk of developing cataracts [25, 26]. Our genetically modified iPSCs propose a platform for further research to create potentially less harmful methods of treating DR while reducing exposure to potentially damaging treatments.

A potential limitation of this research is the re-homing ability of these modified cells, specifically if these cells will have the ability to locate the area that the disease affects. Although our research aims to create cells that can survive in the presence of NK cells, there will be need for further research to determine if they can practically be used in the body to repair vessel damage. Through this creation of

genetically modified iPSCs that are undetectable to the immune system in the treatment of DR, our platform can be used in further studies as a method of testing new drug therapies along with maintaining and repairing the damage to the endothelium lining blood vessels in the retina caused by DR. This research highlights the extensive application of regenerative medicine and the various opportunities it opens for treating diseases like DR.

## Conclusions

This research proposal aims to effectively limit the immunogenicity of the donor-derived iPSCs to establish a universal platform for future studies in DR therapy. The knockout of B2m in HLA Class I and CIITA in HLA Class II combined with the overexpression of CD47 is proposed to limit the immunogenicity of patient-derived iPSCs. This will be tested through comparing the NK-mediated cell death for the unmodified cells and genetically altered endothelial cells. The goal is to show the ability of these modified iPSCs to survive host cell immune responses.

## List of Abbreviations Used

DR: diabetic retinopathy
NK: natural killer
CD47: Cluster of Differentiation 47
HLA: Human Leukocyte Antigen
iPSC: induced pluripotent stem cell
CRISPR-Cas9: clustered regularly interspaced short palindromic repeats and CRISPR-associated protein 9
B2m: Beta-2 microglobulin
CIITA: class II major histocompatibility complex transactivator
PCR: polymerase chain reaction
MANOVA: multivariate analysis of variance

## Conflicts of Interest

The authors declare they have no conflicts of interests.

## Ethics Approval and/or Participant Consent

The study did not require ethics approval or participant consent as it is a research protocol. The research has not been conducted and no participants have been used. The protocol merely outlines the proposed methodology if the research was to be conducted.

## Authors' Contributions

MK: made substantial contributions to the topic idea and methodology, revised the manuscript critically, and gave final approval of the version to be published.
HP: made substantial contributions to the topic idea and methodology, revised the manuscript critically, and gave final approval of the version to be published.
SKS: made substantial contributions to the topic idea and methodology, revised the manuscript critically, and gave final approval of the version to be published.
SL: made substantial contributions to the topic idea and methodology, revised the manuscript critically, and gave final approval of the version to be published.

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