

VOLUME 16 (2022)



# CUSJ

COLUMBIA UNDERGRADUATE SCIENCE JOURNAL



EXPLORING THE  
"CASCADE EFFECT"  
OF AEBSF

ON REDUCED  
GLIOBLASTOMA  
PROLIFERATION

---

## ALSO IN THIS ISSUE

DISCOVERING DENGUE VIRUS ANTIBODY USING PHAGE DISPLAY TECHNOLOGY

CELLULAR AND BIOMATERIAL ENGINEERING APPROACHES FOR TREATING AGE-RELATED MACULAR DEGENERATION

CHALLENGES BETWEEN AND WITHIN RURAL MINORITY DEMOGRAPHICS

---

## Aims and Scope

---

The Columbia Undergraduate Science Journal (CUSJ) was founded in 2006 by students who were passionate about showcasing undergraduate excellence in scientific research. Since then, CUSJ has remained Columbia's premier publication for original scientific research and scholarly reviews, and is managed by an editorial board of undergraduates with a vast scope of interests across all disciplines. The editorial board also manages the Columbia Junior Science Journal (CJSJ), a publication designed to introduce high school students to research and Columbia Scientist, a publication aimed at increasing scientific engagement and thought at all academic levels. In addition to our publications, the CUSJ team is dedicated to fostering the scientific community, both within Columbia and in the surrounding Morningside Heights and Harlem communities. To this end, the board frequently plans outreach and networking events relevant to young and early career scientists, including an annual Research Symposium poster session each spring.

## Copyrights and Permissions

---

Copyright for articles published in the Columbia Undergraduate Science Journal is retained by their authors under a Creative Commons Attribution 4.0 International License. Users are allowed to copy, distribute, and transmit the work in any medium or format provided that the original authors and source are credited. To view a copy of this license, visit [creativecommons.org](http://creativecommons.org)

## Contact

---

Email [cusj@columbia.edu](mailto:cusj@columbia.edu) with any inquiries. Visit [cusj.columbia.edu](http://cusj.columbia.edu) for more information about the journal.

## Administrative

---

|                                      |   |
|--------------------------------------|---|
| Letter from the Editor-in-Chief..... | 3 |
| Letter from the President .....      | 4 |
| Masthead.....                        | 5 |

## Selected Works

---

|  |    |
|--|----|
| <b>Discovering Dengue Virus Antibody Using Phage Display Technology</b><br>Ryeogyeeoung Yoon, Yoonchang Kim, Somin Ahn, Lawrence Kim.....      | 7  |
| <b>Exploring the “Cascade Effect” of AEBSF on Reduced Glioblastoma Proliferation</b><br>Mehwish Mirza.....                                     | 16 |
| <b>Cellular and Biomaterial Approaches for Treating Age-Related Macular Degeneration</b><br>Ashrit Challa, Srikar Sama, Selik Morrishetty..... | 26 |
| <b>Challenges Between and Within Rural Minority Demographics</b><br>Benjamin Blue.....   | 39 |

Dear Readers,

I, along with the rest of the CUSJ board, are ecstatic to present you with the 16th volume of our publication. Since its inception in 2006, CUSJ has strived to promote scientific journalism among undergraduate students and help foster a deeper appreciation and enhanced engagement for everyone in the science community. In this regard, I hope this issue will do exactly that.

This issue encompasses a wide range of topics in hopes that you as a reader will be able to resonate and immerse yourself with the research presented such that you, too, feel provoked to challenge, corroborate, and contribute to the deeply enriching discussions that these articles naturally invite.

This publication is a culmination of a year of hard work from the CUSJ team. Specifically, I would like to thank the editorial board for their input in reviewing and selecting the articles presented in this issue as well as the CUSJ committee who were always so dependable throughout the year and were responsible for editing the articles alongside the authors. Without them, this publication would not have been possible. In addition, I would like to thank our president, Arjun Kudinoor. Throughout the year, Arjun was always able to provide great feedback to improve our processes and was a guiding hand in ensuring that this specific publication lives up to, if not exceeds, the quality that has come to be expected from those before it. I am truly excited for what is to come for the future of CUSJ given Arjun and the rest of the board's dedication to improving and setting up a foundation for CUSJ to succeed in the long-term, which is especially apparent with the inaugural publication of the Columbia Scientist this May led by Aaron Jackson. Lastly, I would like to thank the CUSJ Faculty Advisory Board, whose support has helped us greatly in allowing us to present you with research of the highest standard.

And with that, it is my honor to present you with the 2021-2022 edition of CUSJ. It has been a privilege serving as CUSJ's Editor-in-Chief and I cannot wait to see what is to come!

Thank you so much for reading and supporting CUSJ!

Kazi Rabbe  
Editor-in-Chief, CUSJ  
Columbia Undergraduate Science Journal

Dear Readers,

I am proud to announce the publication of the 2021-22 edition of the Columbia Undergraduate Science Journal! The Columbia Undergraduate Science Journal is our original namesake publication, dedicated to sharing scientific knowledge of the highest quality of scholarship resulting from undergraduate scientific research.

The need for accurate scientific research and reporting is ever-present, especially now more than ever before. A significant issue that plagues society today is misinformation. Opinions uninformed by science present a danger to society - we need not look any further than the still-ongoing COVID-19 pandemic for an example of the consequences of unscientific misinformation. A society misinformed miscalculates. So, in a world of chaotic opinion, we must remain steadfast in our pursuit of scientific knowledge.

Although scientific facts should inform our opinions, it is important to remember that science itself is not a list of facts, but rather a process of discovery. Students all over the world contribute to this collective process as scientific researchers. Through scientific research we explore nature, discover truths, and debate ideas in our relentless pursuit of knowledge. The Columbia Undergraduate Science Journal is a platform for student scientists to share their knowledge so that we may become a better informed society.

It was an honor to read, edit, and review submissions made to our journal. I am grateful to our Columbia Undergraduate Science Journal team and editorial board for their significant contributions to our scientific review process. I am especially grateful to Kazi Rabbe, Editor-in-Chief of the Columbia Undergraduate Science Journal, whose leadership made this publication a success. I would also like to thank the Columbia Undergraduate Science Journal Faculty Advisory Board, a group of esteemed Columbia University professors whose support ensures publication of the highest quality of scholarship.

Congratulations to our authors, and thank you to our readers!

Arjun Kudinoor  
President, Chief Editorial Officer  
Columbia Undergraduate Science Journal

## Editors

|  |   |
|--|---|
| Kazi Rabbe.....CUSJ Editor in Chief                  | Ava Rotondo.....Associate Editor          |
| Arjun Kudinoor.....President                         | Avishi Poddar.....Associate Editor        |
| Shloka Janapaty.....Director of Outreach             | Christine Kim.....Associate Editor        |
| Aaron Jackson.....Columbia Scientist Editor-in-Chief | Davis Hamilton Smith.....Associate Editor |
| Mayeesa Rahman.....CJSJ Editor-in-Chief              | Elisa Huang.....Associate Editor          |
| Vanshika Sriram.....Director of Communications       | Eliza Marie Tagle.....Associate Editor    |
| Chloe Gong.....Director of Events                    | Emma Kaufman.....Associate Editor         |
| Anushka Gupta.....Director of Finance                | Frances Cohen.....Associate Editor        |
| Ellen Ren.....Director of Internal Affairs           | Giovanna Napoleono.....Associate Editor   |
| Arya Roa.....President Emeritus                      | Jimmy Zhang.....Associate Editor          |
| Laurelle Lee Young.....Editor Emeritus               | Kim Lew.....Associate Editor              |
| Isabella Leite.....Editor Emeritus                   | Krystell Santiago.....Associate Editor    |
| Kate Johnson.....Editor Emeritus                     | Lindsey Yang.....Associate Editor         |
| Gloria Charite.....Editor                            | Maria Valerio Roa.....Associate Editor    |
| Mehdi Elfilali.....Editor                            | Neha Mani.....Associate Editor            |
| Teodora Bratu.....Editor                             | Priya Ray.....Associate Editor            |
| Adriana Delagarza.....Associate Editor               | Tyler Shern.....Associate Editor          |
| Andrew Zheng.....Associate Editor                    | Yash Mangalick.....Associate Editor       |
| Anjali Parande.....Associate Editor                  |   |

## Faculty Advisory Board

|   |
|---|
| Hugh Ducklow.....Primary Advisor, Professor of Earth & Environmental Sciences                 |
| Ivana Hughes.....Director of Frontiers of Science & Senior Lecturer in Discipline- Chemistry  |
| Marko Jovanovic.....Assistant Professor of Biological Sciences                                |
| Laura Kaufman.....Professor of Chemistry  |
| Patricia Lindemann.....Director of Undergraduate Studies & Lecturer in Discipline- Psychology |
| Kyle Mandli.....Professor of Applied Mathematics  |
| Gerard Parkin.....Professor of Chemistry  |
| Ron Prywes.....Professor of Biological Sciences   |
| Rachel Rosen.....Associate Professor of Physics   |
| Samuel Sternberg.....Assistant Professor of Biochemistry & Molecular Biophysics               |

## Discovering Dengue Virus Antibody Using Phage Display Technology

Ryeogyeeoung Yoon<sup>1</sup>, Yoonchang Kim<sup>2</sup>, Somn Ahn<sup>3</sup>, Lawrence Kim<sup>4</sup>

<sup>1</sup>Columbia University, New York, NY, USA

<sup>2</sup>Duke University, Durham, NC, USA

<sup>3</sup>Williams College, Williamstown, MA, USA

<sup>4</sup>Georgetown University, Washington, DC, USA

YntoAb Biotech, Seongnam, South Korea

**KEYWORDS:** Dengue virus (NS1), Antibody Discovery, Phage Display, ELISA screening, scFv

**ABSTRACT:** Dengue virus (DENV) is a type of flavivirus transmitted globally by the *Aedes aegypti* mosquito and is the causal agent of Dengue fever. Due to global warming and rising temperatures worldwide, the rate of exposure to DENV is now reaching unprecedented levels, with over 400 million people getting infected each year. Despite worldwide attention to DENV, there is not currently an effective treatment or vaccine. Here, we focused on developing an antibody for the NS1 protein of the DENV, which enables earlier detection of disease and could further decrease the rate of death. Our study utilized a method more time-saving and cost-effective than the traditional method of extracting antibodies from the blood or B-cells of infected animals that show immunity: Phage Display. This technique allowed us to derive monoclonal antibodies (mAbs) from the genome library and select DENV antibodies that bind to antigen proteins. In this study, we used Phage Display to select ninety-six potential antibodies from three phases of panning, which then went through indirect ELISA screening for optical density (OD) confirmation (OD > 1.5). Sixteen of the high optical density antibodies were sent to a DNA sequencing analysis facility where fifteen of them were revealed to be the same 1G1 strain. By understanding how antibodies are discovered using phage display technology, we hope to advance the field with reevaluation of current vaccines and the development of novel ones.

### INTRODUCTION

Dengue is a mosquito-borne viral infection mostly mediated by *Aedes albopictus*. The virus has 4 distinct serotypes (DENV 1-4), which are members of the Flaviviridae family, genus flavivirus. Dengue Virus (DENV), namely DENV 1-4, spreads remarkably well in tropical

regions where high temperatures are maintained all year long. The global temperature increase has resulted in a higher number of tropical and subtropical regions. Previously safe regions have become targets of the DENV, increasing the number of people at risk [1]. These opinions are supported by statistics

gathered by well-known organizations such as the Centers for Disease Control and Prevention (CDC). Their statistics reveal a dramatic increase in the incidence rate of Dengue in recent decades with over 400 million infections, 100 million people showing symptoms, and 22,000 deaths each year [2]. While 22,000 deaths alone are not a statistic to be ignored, we must also watch out for the symptoms of Dengue fever. Most people who are infected by DENV recover within 2 -7 days, accompanied by mild symptoms including nausea, vomiting, and rash. However, severe dengue can result in severe internal bleeding and organ impairment [2].

In order to prevent such symptoms and casualties, it is imperative to diagnose the disease quickly and precisely. Currently, most diagnoses is based on reverse-transcription PCR (RT-PCR), a technique that enables the identification of a patient's serotype of DENV. In addition to diagnosing, we must also find a cure or vaccine for Dengue. The vaccine, Dengvaxia, is currently in use; however, in 2017, the manufacturer announced that even those who have received the vaccine may be at risk of developing severe dengue if they become infected after being vaccinated [3]. As such, finding a more effective vaccine or treatment against DENV has become more important than ever. Conventional methods of vaccine development require the injection of the virus's antigens into experimental animals such as mice and rabbits. Scientists wait for the animals to develop an immune reaction and then extract either the animal's antibodies against the virus from their blood or B cells, which develop the antibodies [4]. Due to the duration of the antibody development in the animal's immune system, this method is time-consuming (usually about 6 months when using rats). Furthermore, we cannot disregard the ethical implications of ani-

mal testing. Therefore, in our study, we offer an alternative –Phage Display technology– in the discovery of an antibody for the non-structural protein 1 (NS1) antigen, a biomarker for DENV.

Phage Display technology refers to a bioengineering technique that allows in vitro selection of monoclonal antibodies (mAbs) by displaying peptides or antibodies on the surface of bacteriophages. Since the whole process occurs outside a living organism in a short timeframe, it is more ethical and time-effective compared to conventional methods [5]. The procedure consists of two main parts: antibody discovery and antibody production. The antibody discovery phase begins with the construction of the phage display library. Once the library is created, the panning process begins, which exposes the library to the target protein – the NS1 protein. Unbound phages are washed away, and those that show affinity for the protein are recovered by elution. The recovered phages are used to infect new host cells for amplification. Repeated panning in the Phage Display technique allows for the selection of only those that maintain a high affinity for the protein, resulting in high specificity of the antibodies. Once the final panning is complete, bounded antibodies go through ELISA screening with a secondary antibody containing an anti-HA tag. Only those with Optical Density (OD) values over 1.5 are considered valuable. The plasmid of these antibodies is separated and sent to sequencing analysis. The antibody production phase began with the cloning of eligible colonies. Consistent human antibody fragments produced through the Phage Display technique can be re-cloned into IgG expression vectors for the generation of antibodies in the human body. Because it maintains compatibility with the human immune system, it should be highly efficacious and safer compared to the conventional method.

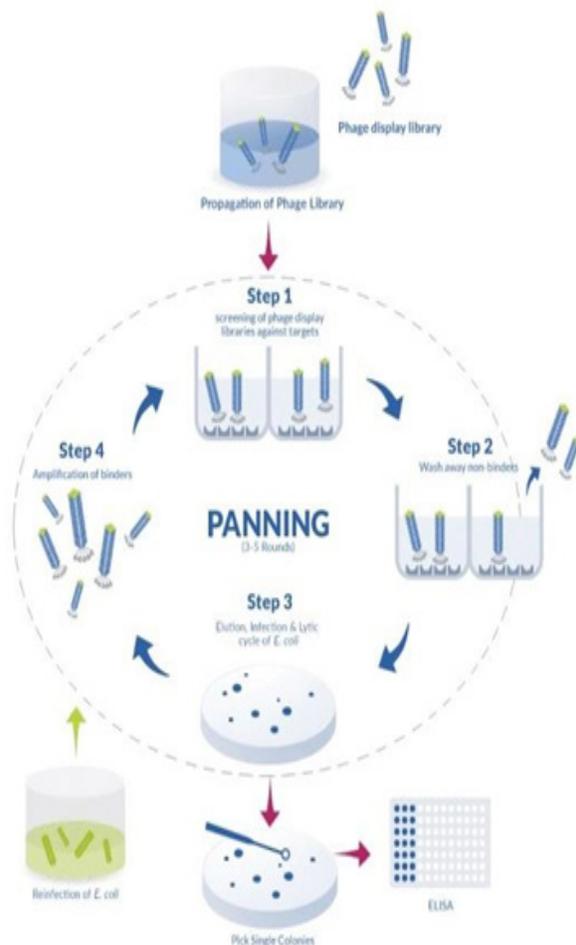


Figure 1. Phage Display Cycle [6]

Over the course of this project, we focused on developing an antibody for the Earth-threatening Dengue virus with a more productive ‘Phage Display’ technique. Discovering the specific sequence of antibodies for the NS-1 protein of Dengue Virus has the potential of manufacturing helpful vaccines or treatments.

## METHODS

### 1. Antigen coating, panning, rescue

Immune tubes containing a coating buffer and protein were prepared prior to the experiment. The panning and rescuing steps were

executed three times each.

Phages and a 3% skim milk 1XPBS solution were added to the tubes. After washing and eluting, the solution was transferred to a tube containing Tris-Cl and bacterial strain TG1. In order to estimate the number of infected bacteria, the cultured bacteria were smeared on a 90mm LB agar plate containing Ampicillin. The remaining infected bacteria were centrifuged and smeared on a 150mm LB agar plate before overnight incubation.

After counting the number of bacteria grown in the 90mm plate, a culture medium was added to the 150mm plate. The mixture was then inoculated in a SB-ampicillin culture medium. Helper phage and Kanamycin were also added before overnight incubation.

To prepare for the third and final panning step, the final medium was centrifuged and added to a tube containing 5XPEG. The supernatant was then filtered and used for panning and rescuing.

### 2. Seeding and induction

The bacteria grown in the 90mm plate were counted. Single colony bacteria were picked from the 90mm plate and seeded into a 96 well plate containing the SB-ampicillin culture medium. After overnight incubation, the single colony bacteria stock was transferred to another 96 well plate containing the SB-ampicillin culture medium and placed in the deep freezer. IPTG, a molecular mimic of allolactose, was added before overnight incubation.

### 3. Screening and seeding

To prepare for screening, periplasmic sup was collected. The incubated single colony bacteria were centrifuged. After removing the supernatant, 1XTES buffer was added to the bacteria pallet, creating a concentration difference. This was kept in ice to extract periplasm

using osmosis. After a 0.2XTES buffer was added and kept in ice, the mixture was centrifuged to collect the periplasmic sup from E.coli in a form of supernatant.

In order to prepare ELISA screening, the protein-coated 96 well plate was blocked with 3% skim milk. 3 columns of the plate were coated with protein and skim milk, while another 3 columns were blocked by skim milk only (for the control). The periplasmic sup was added to each well (6 columns). After 3 times of washing with a wash buffer, a TMB substrate solution was added. TMB reaction was stopped by adding sulfuric acid, and the result was compared with the control. The optical density (O.D) of each well was recorded with an ELISA reader (450nm). The stocks of the wells with positive results ( $1.5 < \text{O.D}$ ) were transferred into different 15mL tubes.

#### 4. DNA prep and Sequencing

The incubated stocks were transferred to 1.5ml tubes, which were then centrifuged. The pallet was filtered and resuspended with P1 buffer (resuspension buffer) [7]. P2 buffer (lysis buffer) was added and mixed by flipping the tubes upside down several times, and it was repeated with P3 buffer (neutralization buffer) [7]. After centrifuging the mixture, the supernatant was transferred into miniprep tube columns. With these columns, the mixture was centrifuged 5 times (first: the mixture itself; second: with EW buffer; third: with PW buffer; fourth: the resultant mixture; fifth: with new tube columns and DW buffer). In order to sequence the DNA collected, they were quantified with a TAKE3 device. These samples were sent to MacroGen and sequenced. Using the online website, Expasy, the sequenced DNA was translated into an amino acid sequence. The website gave several amino acid sequences depending on the starting base to be read as a codon. The frame containing the linker (GQSS

... GGGG) and H6 tag was chosen as the correct sequence.

## RESULTS

### Titerting Results from Panning and Seeding in 90mm plate

To estimate the number of colonies after the 3rd panning, we counted the number of single colonies in the 90mm plate. Table 1 shows the number of colonies counted on each plate (total 4). We divided the section of the plate into eighths and counted each section. Assuming each section maintains a similar number of colonies, we multiplied the number of colonies counted in each section by 8.

### Analyzing the ELISA Result

In order to test the presence of targeted antibodies, we performed ELISA screening on the samples of periplasmic sup. The result of the ELISA test was presented in tables 2A to 2D, each with separate columns for antibody and for 3% skim milk. We compared the columns for antibody to the columns for 3% skim milk which were controls.

After the analysis of the data from the ELISA reader (450nm wavelength), we collected several positive results (blue and yellow cells from Table 2) with an anomaly (C6 in Table 2A). Each table is performed by different people. Stocks with a number greater than 0.2 are highlighted in blue with the gradient (darker blue means the value is greater). Blue samples indicate positive results which means the antibodies are present. 8 yellow stocks were randomly selected for sequencing. The anomaly could have resulted from contamination during pipetting or unsuccessful washing. Based on these results, we randomly chose 8 stocks that gave positive results (Table 2) to quantify the DNA of antibodies before sequencing. 8 randomly selected stocks are colored in yellow in Tables 2A to 2D.

Table 1. Titering Results of Four Plates After the 3rd Panning Process

| Plate number | Number of Colonies | Number of Colonies in 1 $\mu$ L | Number of Colonies in 10ml |
|--------------|--------------------|---------------------------------|----------------------------|
| 1            | 496 x 2            | 1*10 <sup>3</sup>               | 1*10 <sup>7</sup>          |
| 2            | 712 x 8            | 5.7*10 <sup>3</sup>             | 5.7*10 <sup>7</sup>        |
| 3            | 457 x 8            | 3.6*10 <sup>3</sup>             | 3.6*10 <sup>7</sup>        |
| 4            | 537 x 8            | 4.3*10 <sup>3</sup>             | 4.3*10 <sup>7</sup>        |

Table 2. Cultured antibody ELISA Test Result under 450nm wavelength

A)

|   | Antibody |       |       | 3% Skim Milk |       |       |
|---|----------|-------|-------|--------------|-------|-------|
|   | 1        | 2     | 3     | 4            | 5     | 6     |
| A | 0.049    | 0.669 | 0.787 | 0.043        | 0.044 | 0.043 |
| B | 0.043    | 1.577 | 1.386 | 0.044        | 0.045 | 0.045 |
| C | 0.876    | 1.691 | 2.086 | 0.046        | 0.047 | 0.081 |
| D | 0.077    | 1.862 | 0.76  | 0.043        | 0.043 | 0.045 |
| E | 0.046    | 0.135 | 2.039 | 0.044        | 0.043 | 0.045 |
| F | 2.989    | 1.39  | 1.833 | 0.043        | 0.043 | 0.045 |
| G | 2.023    | 1.895 | 0.1   | 0.043        | 0.047 | 0.043 |
| H | 0.044    | 2.309 | 2.893 | 0.053        | 0.045 | 0.044 |

B)

|   | Antibody |       |       | 3% Skim Milk |       |       |
|---|----------|-------|-------|--------------|-------|-------|
|   | 1        | 2     | 3     | 4            | 5     | 6     |
| A | 0.054    | 1.515 | 0.416 | 0.045        | 0.046 | 0.044 |
| B | 1.453    | 0.045 | 0.051 | 0.044        | 0.067 | 0.044 |
| C | 0.052    | 0.046 | 0.043 | 0.045        | 0.05  | 0.044 |
| D | 0.046    | 1.695 | 0.042 | 0.051        | 0.046 | 0.082 |
| E | 0.305    | 1.459 | 0.045 | 0.044        | 0.043 | 0.042 |
| F | 0.043    | 0.044 | 0.043 | 0.044        | 0.045 | 0.043 |
| G | 0.044    | 0.047 | 0.044 | 0.044        | 0.044 | 0.047 |
| H | 0.045    | 0.043 | 0.043 | 0.045        | 0.048 | 0.046 |

C)

|   | Antibody |       |        | 3% Skim Milk |       |       |
|---|----------|-------|--------|--------------|-------|-------|
|   | 1        | 2     | 3      | 4            | 5     | 6     |
| A | 0.062    | 2.262 | 1.941  | 0.044        | 0.043 | 0.049 |
| B | 0.05     | 0.044 | 0.046  | 0.047        | 0.044 | 0.047 |
| C | 0.393    | 0.045 | 1.758  | 0.045        | 0.045 | 0.044 |
| D | 0.047    | 0.78  | 0.2797 | 0.047        | 0.046 | 0.045 |
| E | 1.869    | 0.115 | 0.049  | 0.046        | 0.065 | 0.045 |
| F | 0.225    | 0.049 | 1.456  | 0.044        | 0.044 | 0.047 |
| G | 0.045    | 0.046 | 0.056  | 0.046        | 0.046 | 0.049 |
| H | 2.684    | 0.055 | 0.064  | 0.046        | 0.045 | 0.048 |

D)

|   | Antibody |       |       | 3% Skim Milk |       |       |
|---|----------|-------|-------|--------------|-------|-------|
|   | 1        | 2     | 3     | 4            | 5     | 6     |
| A | 0.043    | 0.043 | 0.491 | 0.045        | 0.042 | 0.043 |
| B | 0.045    | 0.044 | 0.778 | 0.044        | 0.043 | 0.043 |
| C | 0.044    | 0.044 | 0.043 | 0.044        | 0.045 | 0.043 |
| D | 0.045    | 0.389 | 0.043 | 0.043        | 0.046 | 0.044 |
| E | 2.034    | 0.045 | 0.25  | 0.043        | 0.043 | 0.044 |
| F | 2.152    | 0.046 | 0.044 | 0.044        | 0.045 | 0.044 |
| G | 0.044    | 0.044 | 1.964 | 0.043        | 0.044 | 0.043 |
| H | 0.043    | 0.044 | 1.997 | 0.044        | 0.043 | 0.044 |

### DNA Quantification before Sequencing

The stocks that have absorbance ratios of 260 nm/280 nm above 1.8~2 were considered to have sufficient amounts of DNA for successful sequencing [8]. With the Take3 micro-volume plate, the absorbance ratio (260nm/280nm) of each sample was collected (Table 3). Samples were randomly selected (8 stocks which are yellow cells in Figure 2). The highlighted number indicates the concentration of DNA.

There were variances in the value

of the DNA quantities per microliter. It was expected to have no variance as the samples were prepared with the same method and the environment. These variances may indicate that the samples were not pure or the sequencing was unsuccessful. However, because the observations at 260 nm were greater than those at 280 nm, resulting in high DNA to protein ratios (greater than 2), we assumed that the DNA samples were sufficiently pure to be sequenced.

### Analyzing the Sequencing Results

After the DNA quantification, we requested Humanizing Genomics Macrogen to sequence the 8 samples. The purpose of single chain Fragment variable (scFv) sequencing was to understand the different types and positions of amino acids within the created antibody. After obtaining the plasmid of the single colony antibodies, each DNA strand was sequenced using the 5', 3'-oligonucleotide of a scFv. The final order of the scFv was determined after reading the sequence from both directions and combining the common regions. (ABI3730XL, Sanger-method sequencing, Phred Score ≥20). In order to analyze the results of the sequencing, we used an ExPASy(<https://web.expasy.org/translate/>) that translated the DNA sequence in our raw data (Figure 2) into corresponding amino acids. From the results, we determined the accurate reading frame (Figure 3) by identifying the H6 tag and the linker. Within the scFv, there is a light chain variable region followed by a linker and a heavy chain variable region. As heavy and light chains of the antibodies were linked with linker protein (gggssggggs) at heavy chain 6, the presence of the linker and H6 tag on the amino acid sequence indicates that we have successfully discovered our targeted antibody.

```
>H210802-040_M16_1G1_pcd-forward.ab1 1512
GGACTAAAATTAATGAAAAGACAGCTATCGCGATTGCGAGTGGCACTGGCT
GGTTTCGCTACCGTGGCCAGCGGCCCTGACTCAGCCGCTCTCGGGTGC
AGCAACCTGGGAGAACCGTCAAGATAACCTGCTCGGGAGTACTGGTG
GCTATGGCTGGTATCAGCAGAAGTCACTGGCAGTGGCCCTGTCACTCTG
ATCTATGACAACCAACAGACCCCTCGGACATCCCTCAGCATTCCTCCGG
TTCCAATCCGGCTCCACAGCCACATTAACCATCACTGGGGTCCAGCCG
AGGACGAGGCTATCTATTTCTGGTGGCTACGACAGCAGTAGTGATAGT
GGTATATTTGGGGCCGGGACACCCCTGACCGTCTAGGTGAGTCTCTAG
ATCTTCGGCGGGTGGTGGCAGCTCCGGTGGTGGCGGTTCGGCCGTGACGT
TGGACGAGTCCGGGGCCGGCTCCAGACCGCCGGAGGAGCGCTCAGCCCT
GTCTGCAAGGCCTCCGGGTTGCACTTCAGCAGTTATGAGATGCACTGGGT
GCGACAGGCGCCCGGCAAGGGTTGGAGTTTCGTCGCTGGTATTGGCAACA
CTGGTAGTAGCACAGCATACGGGCGCGGTGAAGGGCCGTGCCACATC
TCGAGGGACACCGGCAGAGCACACTGAGGCTGCAAGCTGAACAACTCAG
GGCTGAGGACACCGGCACCTACTACTGCGCCAAAACACTGGTAGTGGCT
ATTGTGATATTTTACTGATTACAGTGTGTGGGGTTATAGTACTGGTAAC
ATCGACGCATGGGGCCACGGGACCGAAGTCACTCGTCTCCTCCACTAGTGG
CCAGGCGGGCCAGCACCATCACCATCACCATGGCGCATACCCGTACGACG
TTCCGGACTACGCTTCTTAGGAGGGTGGTGGCTCTGAGGGTGGCGGTCT
GAGGGTGGCGGCTCTGAGGGAGGGCGGTTCGGTGGTGGCTCTGGTTCGGG
TGATTTTGATTATGAAAAGATGGCAAACGCTAATAAGGGGGCTATGACCC
AAAATGCCGATGAAAACGTGCTACAGTCTGACGCTAAAGGCAAACTTGAT
TCTGTCGCTACTGATTACGGTGGCTGCTATCGATGGTTCATTGGTGACGTT
TCCGGCTTGTAGGGATATGGTCTACGGGTGATTTGCTGGCTCAAT
CCCAATGGCTCAAGTCCGGGACGGGATAATTCCTTTAAGAAAATTCGG
CAAATTACCTCCCCCCCCAACCGGTGAAGGCCCCCTTTCTTTGGGCGGG
AAACATAGAATTTTTTGGGAAAAAAAACCTTTCCGGGGGTTTGGTTT
TTTTTTTTGGCCCTTTTTTTTTTTTTTTTTCATTAACACCGCAAAAAGAAA
CACAAAATAAAGCCCGCCCGGGGAGCGGGGGGAAAAGATTTAT
TTTTTTTTTTTTTTTTTTTTTTTTTTTTCTCTCTCTCCCTCCCTCCCTCC
```

Figure 2. Raw DNA Sequence

Table 3. DNA Quantification Results of 8 Antibody Stocks Randomly Selected

|   | 2 | 3              |         |
|---|---|----------------|---------|
| A |   | SPL1           | Well ID |
|   |   | 0.063          | 260     |
|   |   | 0.031          | 280     |
|   |   | 2.055          | 260/280 |
|   |   | <b>63.015</b>  | ng/μl   |
| B |   | SPL2           | Well ID |
|   |   | 0.055          | 260     |
|   |   | 0.025          | 280     |
|   |   | 2.18           | 260/280 |
|   |   | <b>55.241</b>  | ng/μl   |
| C |   | SPL4           | Well ID |
|   |   | 0.033          | 260     |
|   |   | 0.019          | 280     |
|   |   | 1.703          | 260/280 |
|   |   | <b>33.008</b>  | ng/μl   |
| D |   | SPL6           | Well ID |
|   |   | 0.163          | 260     |
|   |   | 0.076          | 280     |
|   |   | 2.159          | 260/280 |
|   |   | <b>163.393</b> | ng/μl   |
| E |   | SPL8           | Well ID |
|   |   | 0.116          | 260     |
|   |   | 0.053          | 280     |
|   |   | 2.182          | 260/280 |
|   |   | <b>116.458</b> | ng/μl   |



Figure 3. Accurate Translated Reading Frame

## DISCUSSION

We demonstrate here the successful discovery of an antibody for the non-structural protein 1 (NS1) antigen, a biomarker of DENV. Our discovery employed Phage Display Technology, which enabled in vitro selection of monoclonal antibodies by presenting antibodies on the surface of bacteriophages.

At large, our results indicate that discovered antibodies are highly specific to the targeted NS1 antigen. scFv sequencing confirmed our accuracy with the identified H6 tag and linker on our Amino acid sequence. The high specificity of the antibody was derived from the ELISA technique and the quantity of DNA in the cultured colony was verified through DNA quantification prior to sequencing. Clones that had an OD value above 1.5 in the ELISA technique were only allowed in cultivation and colonies that maintained an absorbance ratio (260nm/280nm UV light) above 2 were considered pure enough to be tested.

Due to an increasing number of DENV patients concurrent with global warming, the necessity for therapeutics to overcome this crisis is on the rise. The ultimate goal of our study was to aid the development of effective vaccines or treatments for DENV; thus, enabling

earlier detection of the disease and further lowering the rate of death.

Clearly, further studies are warranted to elucidate the immune parameters that could suggest a solution to overcome the limitation of the current vaccine (Dengvaxia). Unlike the current vaccine, the novel vaccine derived from our NS1 Protein antibody is expected to significantly lessen the possibility of re-infection of Dengue Variants. In prospective studies, the sequence of the antibody should be investigated further to figure out the adequate sequence of DNA that could potentially neutralize the targeted antigen. We thereby anticipate yielding efficient antibody therapeutic from the antigen-specific neutralizing sequence.

## AUTHOR INFORMATION

### Corresponding Author

\*Ryeogyeeoung Yoon  
ry2410@columbia.edu

### Competing Interests

The authors declare no competing financial and non-financial interests.

## ACKNOWLEDGEMENTS

We thank YntoAb for providing us with an equipped laboratory.

## ABBREVIATIONS

DENV- Dengue Virus  
1G1- Histone H3 Antibody  
mAbs- Monoclonal Antibodies  
NS1- Nonstructural protein 1  
scFv- Single-chain Variable Fragment  
OD- Optical Density  
ELISA- Enzyme-linked Immunoassay  
IPTG- Isopropyl  $\beta$ -D-1-thiogalactopyranoside

## REFERENCES

- [1] Guzman, M., Halstead, S., Artsob, H. et al. (2010). Dengue: a continuing global threat. *Nat Rev Microbiol* 8, S7–S16 . <https://doi.org/10.1038/nrmicro2460>
- [2] Centers for Disease Control and Prevention. (2019). About Dengue: What you need to know. CDC 24/7: Saving Lives, Protecting People. <https://www.cdc.gov/dengue/about/index.html>
- [3] World Health Organization. (2021). Dengue and severe dengue. WHO Press, Geneva, Switzerland. <https://www.who.int/news-room/fact-sheets/detail/dengue-and-severe-dengue>
- [4] S.Kellermann, L.Green.(2002). Antibody discovery: the use of transgenic mice to generate human monoclonal antibodies for therapeutics. *Current Opinion in Biotechnology*. 13 (6)., 593-597,.
- [5] J.Bábíčková, L'.Tóthová, P.Boor, P. Celec. (2013). In vivo phage display - A discovery tool in molecular biomedicine. *Biotechnology Advances*.,31 (8), 1247-1259,.
- [6] Proteogenix. Phage display cycle: phage display library, panning/biopanning and validation by ELISA. Proteogenix.,Schiltigheim, France. <https://www.proteogenix.science/antibody-production/phage-display-services/>
- [7] DNA-spin Plasmid DNA Purification Kit from iNtRON Biotechnology
- [8] Olson, N. D., & Morrow, J. B. (2012). DNA extract characterization process for microbial detection methods development and validation. *BMC research notes*, 5, 668

## Exploring the “Cascade Effect” of AEBSF on Reduced Glioblastoma Proliferation

Mehwish Mirza

Columbia University, New York, NY, USA

**KEYWORDS:** Glioblastoma, AEBSF, V-ATPase, Cascade Effect, Metalloproteinases, Cancer Proliferation, Extracellular Matrix Disassembly in Cancer

**ABSTRACT:** Glioblastomas (GBMs) are one of the deadliest cancers, and yet still lack treatment. In this study, serine protease inhibitor AEBSF is shown to decrease GBM viability and proliferation through a series of intertwined steps. By decreasing the expression of ATP6V1A--the first step of the “cascade effect”--AEBSF creates an unfavorable environment for protease activity. Such “unfavorability” is proven by a decline in expression of proteases MMP9 and ELANE upon GBM treatment with AEBSF. Increasingly higher concentrations of AEBSF are shown to decrease the percentage of cell viability, proliferation, and metastasis. This is explained through biological process enrichment analyses, which indicate that MMP9 and ELANE play a role in the disassembly of the extracellular protein network. This disassembly of the extracellular environment enables tumorigenic spread and viability. The results of the viability and proliferation assays, in combination with those of the indirect ELISAs, enrichment analyses, and scratch assays, underscore an intricate and profound sequence of events that constitute the “cascade effect”.

### INTRODUCTION

Glioblastomas (GBM) are grade IV gliomas and are the most lethal primary malignancy of the central nervous system (CNS). Despite comprising 54% of all gliomas and 16% of all primary brain tumors, GBM lacks a cure and only has a handful of treatments [1]. The lack of curative treatment for GBM can be attributed to several factors, some of which being high GBM resistance rates, rapid angiogenesis, and vast spread [10].

Studies have demonstrated that, unlike non-tumorigenic cells – which have extracellular pH (pHe) of approximately 7.4 – GBM have acidic pHe ranging between 6.7-7.1 [3].

This acidic extracellular pH has recently been demonstrated to activate proteases (such as serine proteases, plasminogen activators, and metalloproteinases). These proteases degrade the GBM extracellular environment, explaining why they have been indicated to be involved in GBM growth, invasion, angiogenesis and metastasis [2, 4]. Given the importance of pH in GBM progression, this study aimed to focus on pH regulation as a means of GBM treatment.

A major regulator of physiological pH is the vacuolar (H<sup>+</sup>)-ATPase (V-ATPase). V-ATPases are ubiquitously expressed and can be found in lysosomes,

endosomes, secretory vesicles, clathrin-coated vesicles, and the plasma membrane [4]. Consisting of 13 subunits in mammalian cells, the primary function of these proton pumps is to regulate both intracellular and extracellular acidification [5]. V-ATPases comprise two domains: the V0 and V1 domains. The peripheral V1 domain consists of eight subunits (A–H), all of which assist in ATP hydrolysis. The membrane-embedded V0 domain—consisting of subunits a, c, c', d, and e—is responsible for proton translocation [6]. In this study, subunit A of the V1 domain (ATP6V1A) is closely examined due to its cytoplasmic location and ubiquitous nature across the body [14, 15].

### Discovering the "Cascade Effect" of AEBSF in Glioblastoma Metastasis

#### Mechanics of a "Normal" Glioblastoma Cell

Plasma membrane V-ATPases are overexpressed, producing abnormally acidic extracellular environments favorable for the functioning of PLAU. Extracellular PLAU activates MMP9, which degrades the ECM and thereby promotes tumor metastasis.

#### Mechanics of a Glioblastoma Cell Treated with AEBSF

AEBSF decreases extracellular PLAU, thereby decreasing MMP9 activation. This, in turn, reduces ECM degradation and tumor metastasis.

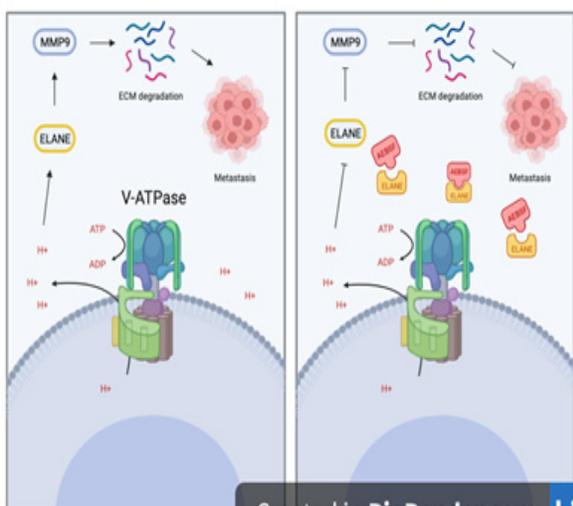


Figure 1. Created by author

This study explores the sequence of events in the “cascade effect” perpetuated by 4-benzenesulfonyl fluoride hydrochloride (AEBSF). AEBSF, also known as AEBSF hydrochloride, is an irreversible serine protease inhibitor that has previously been indicated to inactivate serine proteases such as chymotrypsin and proteinase K [11]. AEBSF has been shown in literature to have a potential regulatory effect on the secretion of antitumor effector molecules in leukemia [12]. AEBSF has also been shown to reduce dengue virus infection via decreased cholesterol synthesis [13].

In this study, AEBSF was studied under the context of GB, proliferation and metastasis. The question sought to be answered is, “What are the chronological events that occur in the reduction of GBM metastasis perpetuated by AEBSF?”

The findings in this study may: 1) provide more insight into the mechanics of GBM metastasis, 2) highlight the role of serine proteases in GBM progression, and 3) provide greater insight into such proteases’ mechanisms of action, thereby clarifying the link between ECM disassembly and GBM metastasis.

## METHODS

### Cell Culture

T98G human glioblastoma cells were obtained from the American Type Culture Collection (CRL-1690; ATCC, Manassas, VA, USA). Cells were grown in Dulbecco’s modified Eagle medium (DMEM) supplemented with 1% 5000 U/mL Penicillin-Streptomycin (ThermoFisher, Waltham, MA, USA) and 10% fetal bovine serum (FBS) (Gibco, Waltham, MA, USA). Cells were incubated at 37 °C with 5% CO<sub>2</sub> and 100% humidity. Cell culture images were taken with a Nikon Eclipse TS100 light microscope at 100x magnification.

#### *Reagent preparation*

AEBSF (solubility 50mg/mL H<sub>2</sub>O, molar mass: 239.69 g/mol) was dissolved in H<sub>2</sub>O.

#### *Treatment of cells with AEBSF*

Cells were seeded at  $\sim 10^6$  cells in T25 flasks for 24 hours (incubated at 37°C with 5% CO<sub>2</sub> and 100% humidity). Cells were subsequently treated with the appropriate AEBSF concentrations--125 $\mu$ M, 500 $\mu$ M, or 2000 $\mu$ M--for 48 hours.

#### *Preparation of lysates for enzyme-linked immunosorbent assay (ELISA)*

Phosphate buffered saline (PBS) (ThermoFisher, Waltham, MA, USA), 1X cell lysis buffer (Cell Signaling Technology, Danvers, MA, USA), and protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA) were used to lyse  $10^6$  cells/mL from each treatment group. Cells were incubated at 37°C with 5% CO<sub>2</sub> and 100% humidity with trypsin for 3 minutes until detachment. Cells were subsequently neutralized with DMEM and transferred to 15mL centrifuge tubes. Tubes were gently inverted and cells were counted using ViCell XR (Beckman Coulter, Indianapolis, IN, USA). Tubes were subsequently centrifuged at 1000 rpm for 7 minutes at room temperature, after which the pellets were poured off and the pellet was broken. Chilled PBS was added to the tubes, which were subsequently re-centrifuged at 1000 rpm for 7 minutes at 25°C. The residual supernatant was poured out and the pellet was broken up. The appropriate volume of lysis buffer, calculated using the following equation, was added to each tube and incubated on ice for 10 minutes:  $[\text{Viable cell count (cells/mL)} \times \text{volume of cell suspension prior to first round of centrifugation (mL)}] \div 106 \text{ cells/mL}$

Lysates from the tubes were subsequently transferred to chilled Eppendorf tubes.

The tubes were micro-centrifuged at 13000 rpm for 15 minutes at 4°C. The supernatants were stored at -80°C.

#### *Indirect ELISA*

100 $\mu$ L/well of lysates were transferred at 4 replicates per treatment group to a 96-well plate. Lysates were left to incubate for 24 hours at 4°C and then removed by blotting. 200 $\mu$ L/well of blocking buffer (ThermoFisher, Waltham, MA, USA) with 1% bovine serum albumin (BSA) (Seracare, Milford, MA, USA) was added and left to incubate for 10 minutes at 25°C.

#### *Cell proliferation assay*

Cells were seeded at 62,500 cells/well 24 hours prior to treatment in a 6-well plate. Cells were subsequently treated for 48 hours, supernatants were collected, and cell proliferation was analyzed using a synergy machine.

#### *Trypan Blue Exclusion Viability Assay*

Cells were seeded at  $\sim 40,000$  cells/flask 24 hours prior to treatment. Cells were subsequently treated for 48 hours, supernatants were collected, and cells were trypsinized and counted using ViCell XR (Beckman Coulter, Indianapolis, IN, USA).

#### *Reverse Docking*

Reverse docking tool ChemMapper was used to predict the protein targets of AEBSF. The SMILES (Simplified Molecular Input Line Entry System) notation of AEBSF, obtained from PubChem, was inputted into ChemMapper. Subsequently, the "Target Navigator" service was selected and the 3D similarity method SHAFT was chosen. The default similarity threshold of 1.2 was selected, and the Bioactivity Database ChEMBL was then chosen [7-9].

### *Data Analysis*

Statistical and computational biology analyses were performed using Microsoft Excel. An unpaired, two-tailed T-test was performed in Excel, and a p-value < 0.05 was considered statistically significant. Means and standard deviations are taken for all data and compared as % of control.

## RESULTS

### *AEBSF reduces T98G proliferation*

AEBSF was first demonstrated to reduce proliferation in T98G cells. As hypothesized, AEBSF reduces proliferation in a statistically significant manner, demonstrating the efficacy of this treatment (Fig. 1A).

### *AEBSF decreases T98G viability*

AEBSF was also demonstrated to reduce viability in T98G cells. Specifically, higher concentrations of AEBSF were shown to decrease the number of viable cells and increase the number of dead cells (Fig. 2). As hypothesized, AEBSF reduces viability in a statistically significant manner, once again demonstrating the efficacy of this treatment (Fig. 2).

### *Plasma membrane V-ATPase subunit A (ATP6V1A) protein expression is enhanced in glioblastomas and decreased upon AEBSF treatment*

V-ATPase subunit A (ATP6V1A) plays a vital role in the primary function of the overall proton pump--acidification of the extracellular environment. Through an indirect ELISA, it is demonstrated that ATP6V1A protein expression decreases with increasing AEBSF concentration (Fig. 3).

### *AEBSF targets serine proteases and metalloproteinases*

AEBSF is a well known serine protease

inhibitor. Although it is known that AEBSF inhibits its serine proteases such as trypsin and chymotrypsin, shape-screening tool ChemMapper was utilized to predict its protein targets with the highest confidence scores. ChemMapper is fundamentally rooted in the “chemical similarity principle”, which states that structurally similar compounds have similar bioactivities [7]. Thus, it can be said that a higher similarity score between the query ligand (AEBSF in this study) and the database ligands indicates a higher likelihood that a given protein target of the database ligand is also a target of AEBSF, the query ligand. Thus, as previously discussed, a similarity threshold of 1.2 (on a scale of 2.0) was set in order to filter out protein targets of database ligands with low-similarity scores. Using ChemMapper, it was predicted that AEBSF targets 6 serine proteases (Fig. 4A) and 2 metalloproteinases (Fig. 4B).

### *Serine proteases and metalloproteinases targeted by AEBSF primarily play a role in extracellular matrix disassembly and cell migration in glioblastomas*

The eight protein targets predicted by ChemMap were plugged into “Search Tool for the Retrieval of Interacting Genes/Proteins” (STRING) to carry out an enrichment analysis of the biological processes that they are involved in. The top five biological processes, along with their false discovery rates (FDRs) and strength values, were analyzed (Fig. 5).

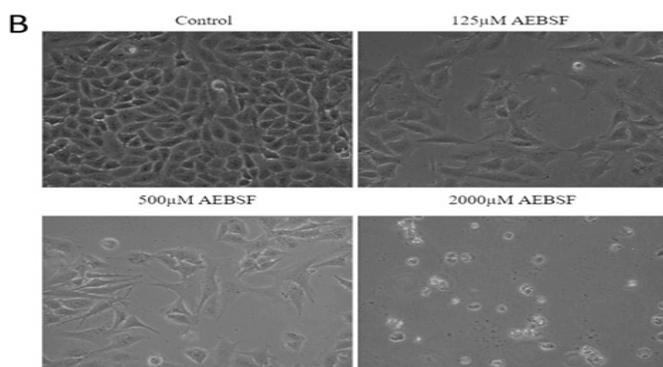
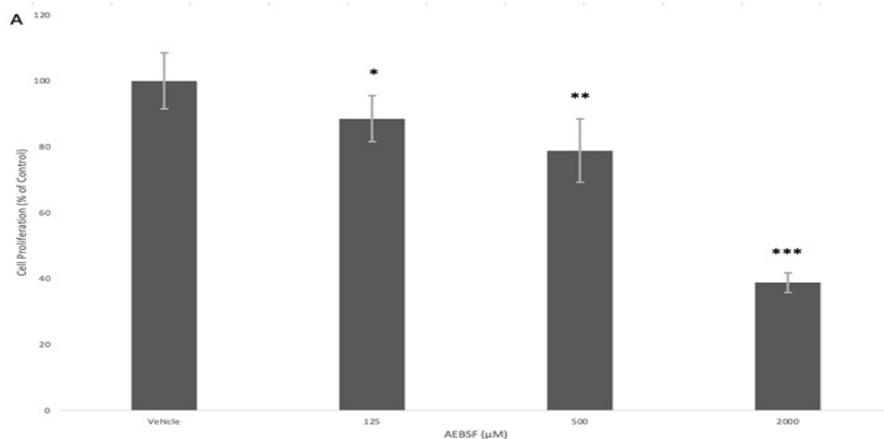
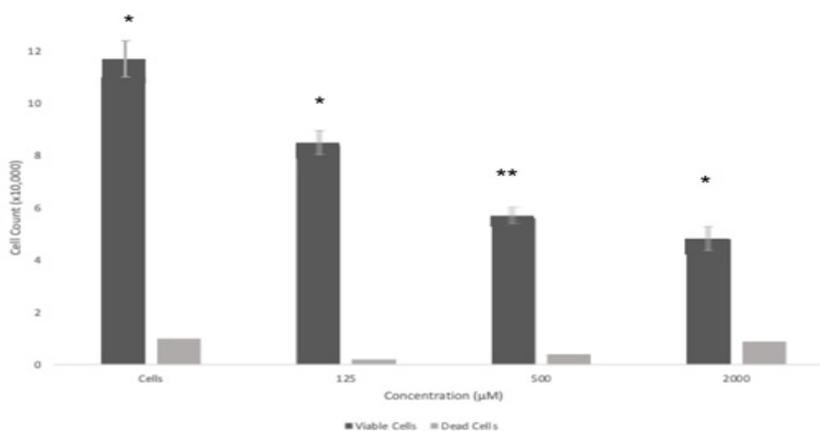


Figure 1. 48-hour treatment with 4-benzenesulfonyl fluoride hydrochloride (AEBSF) reduces proliferation in T98G cells. A: Bars are means  $\pm$  STDEV (n=4). \*Significantly different by t-test at  $p < 0.05$  comparing data to that at vehicle control (cells and H<sub>2</sub>O). \*\*Significantly different by t-test at  $p < 0.01$  comparing data to that at vehicle control. \*\*\*Significantly different by t-test at  $p < 0.001$  comparing data to that at vehicle control. B: Light microscopy images of T98G treated with 125μM, 500μM, or 2000μM AEBSF at 100x.

Figure 2. 48-hour treatment with 4-benzenesulfonyl fluoride hydrochloride (AEBSF) reduces viability in T98G cells.



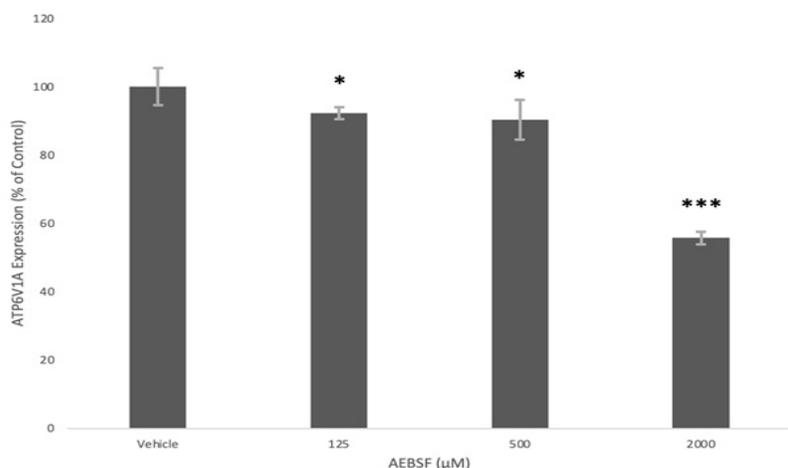
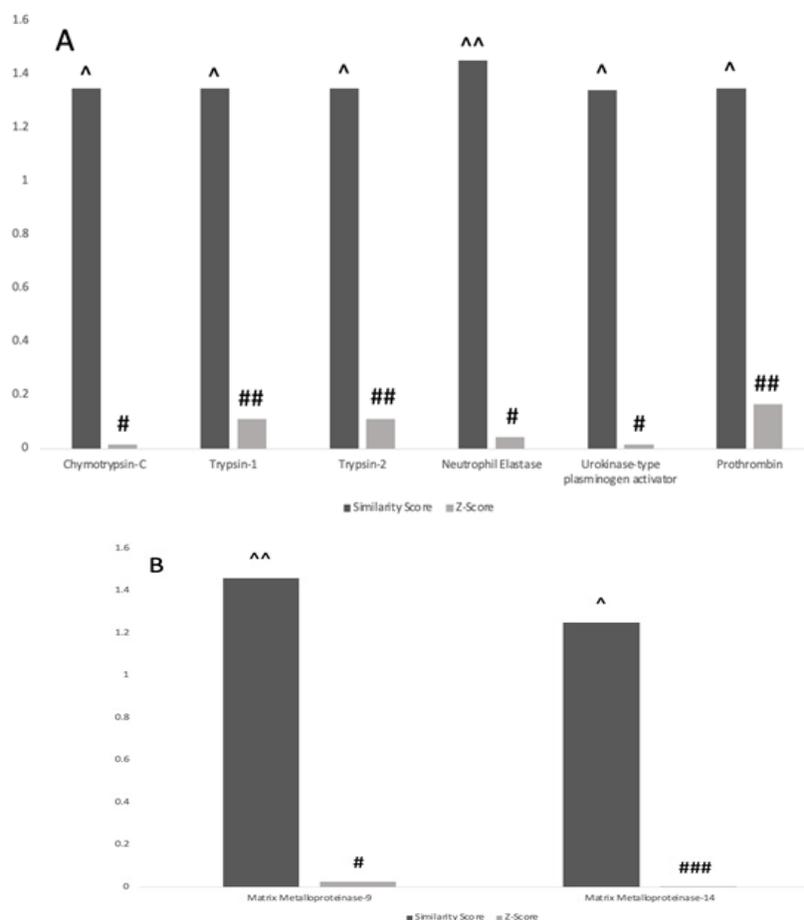


Figure 3. ATP6V1A ELISA on T98G cells treated with 4-benzenesulfonyl fluoride hydrochloride (AEBSF) for 48 hours. 48-hour treatment with AEBSF reduces ATP-6V1A protein expression in T98G.

Figure 4. Reverse docking analysis of 4-benzenesulfonyl fluoride hydrochloride (AEBSF) using ChemMapper. Z-scores and similarity scores of each predicted target are displayed. The similarity score ranges from [0, 2]. The closer the score is to 2.0, the higher the potential of pharmacological association there is between AEBSF and the database ligands of the outputted protein targets ^Similarity score ranges between 1.2-1.4 ^^Similarity score ranges between 1.4-1.6 #Z-score<0.5 ##Z-score<0.1 ###Z-score<0.01 A: AEBSF targets serine proteases Chymotrypsin-C, Trypsin-1, Trypsin-2, Neutrophil Elastase, Urokinase-type plasminogen activator, and Prothrombin. B: AEBSF targets matrix metalloproteinase-9 (MMP9) and matrix metalloproteinase-14 (MMP14).



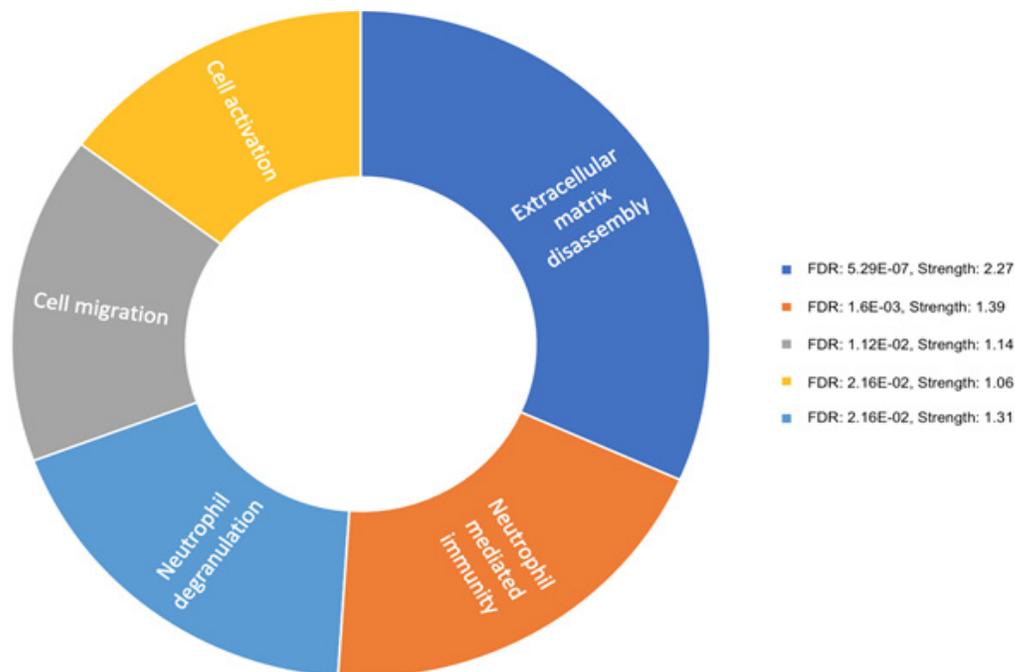


Figure 5. Biological process enrichment analysis of AEBSF serine protease (Chymotrypsin-C, Trypsin-1, Trypsin-2, Neutrophil Elastase, Urokinase-type plasminogen activator, Prothrombin) and metalloproteinase (MMP9, MMP14) targets. Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) was utilized for this analysis. False discovery rates (FDRs) and strength values are displayed;  $p = 7.61e-1$ . Five biological processes with the highest strength values are displayed.

***AEBSF downregulates ELANE and MMP9 protein expression***

Following the reverse docking and biological processes enrichment analyses, neutrophil elastase (ELANE) and matrix metalloproteinase-9 (MMP9) expression were selected to be analyzed. These two proteins were selected to be studied, as in the reverse docking analysis, ELANE and MMP9 were demonstrated to have the highest similarity scores, which was previously established to be a good indicator of the likelihood that a given protein target of the database ligand is also a target of AEBSF, the query ligand (Fig. 4A, 4B). Hence, serine protease ELANE and metalloproteinase MMP9

expression were analyzed through indirect ELISAs. Both ELANE and MMP9 protein expression were demonstrated to decrease with increasing AEBSF concentrations (Fig. 6A, 6B).

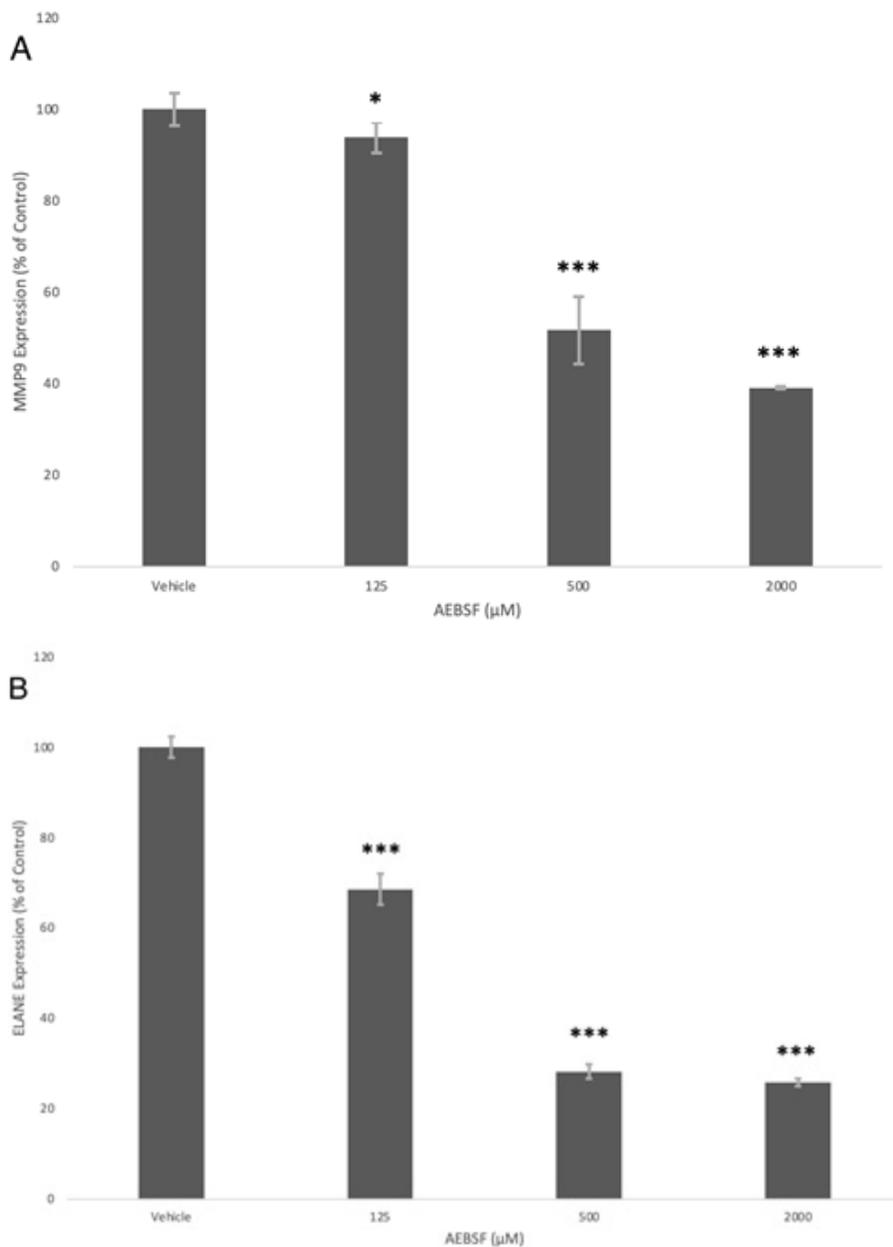


Figure 6. MMP9 and ELANE ELISAs on T98G cells treated with 4-benzenesulfonyl fluoride hydrochloride (AEBSF). Bars are means  $\pm$  STDEV (n=4). A: MMP9 ELISA on T98G cells treated with AEBSF for 48 hours. 48-hour treatment with AEBSF reduces MMP9 protein expression in T98G. B: ELANE ELISA on T98G cells treated with AEBSF for 48 hours. 48-hour treatment with AEBSF reduces ELANE protein expression in T98G.

## DISCUSSION

This project sought to explore the sequence of events of the “cascade effect” perpetuated by AEBSF in GBM. Through an indirect ELISA, it was demonstrated that serine protease inhibitor AEBSF decreases ATP6V1A expression, thereby activating ELANE and MMP9 proteases. This, in turn, was indicated to stimulate ECM degradation and overall GBM metastasis and migration (Fig. 7). It was demonstrated that higher AEBSF concentrations had a greater effect on the studied parameters. However, the optimal AEBSF dosage remains to be determined and will be studied in the future.

In the future, the mechanisms of ELANE and MMP9-mediated ECM disassembly will be studied. This may give greater insight into these proteases’ mechanisms of action, and possibly further clarify the link between ECM disassembly and GBM metastasis.

The findings in this study may provide more insight into the mechanics of GBM metastasis, highlighting the role of serine proteases and tumor metastasis.

## AUTHOR INFORMATION

### Corresponding Author

\*Mehwish Mirza (Academy for Medical Science Technology, Department of Cellular Biology Research, Bergen County Academies, Hackensack, NJ, USA) mehwish.mirza05@gmail.com

### Author Contributions

Mehwish Mirza performed the data analysis and produced the figures and manuscript. Dr. German Sabio provided technical assistance with analysis and pipelines. Dr. German Sabio and Donna Leonardi added project direction and provided guidance. Elisa Huang and Christine Chow provided expert feedback for the project.

### Competing Interests

The authors declare no competing financial and non-financial interests.

## REFERENCES

- [1] Tamimi AF, Juweid M. Epidemiology and Outcome of Glioblastoma. In: De Vleeschouwer S, editor. Glioblastoma [Internet]. Brisbane (AU): Codon Publications; 2017 Sep 27. Chapter 8. doi: 10.15586/codon.glioblastoma.2017.ch8. <https://www.ncbi.nlm.nih.gov/books/NBK470003/>
- [2] Kato, Y., Ozawa, S., Miyamoto, C., Mae-hata, Y., Suzuki, A., Maeda, T., & Baba, Y. (2013). Acidic extracellular microenvironment and cancer. *Cancer cell international*, 13(1), 89. doi: 10.1186/1475-2867-13-89. <https://cancerbiomedcentral.com/articles/10.1186/1475-2867-13-89>
- [3] Ward C, Meehan J, Gray ME, Murray AF, Argyle DJ, Kunkler IH, et al. The impact of tumour pH on cancer progression: strategies for clinical intervention. *Explor Target Antitumor Ther.* 2020;1:71-100. doi: 10.37349/etat.2020.00005. <https://www.explorationpub.com/Journals/etat/Article/10025>
- [4] Park, K. C., Dharmasivam, M., & Richardson, D. R. (2020). The Role of Extracellular Proteases in Tumor Progression and the Development of Innovative Metal Ion Chelators that Inhibit their Activity. *International journal of molecular sciences*, 21(18), 6805. doi: 10.3390/ijms21186805. <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7555822/>
- [5] L. Shannon Holliday, "Vacuolar H<sup>+</sup>-ATPase: An Essential Multitasking Enzyme in Physiology and Pathophysiology", *New Journal of Science*, vol. 2014, Article ID 675430, 21 pages, 2014. doi: 10.1155/2014/675430. <https://down->

- [6] Cotter, K., Capecci, J., Sennoune, S., Huss, M., Maier, M., Martinez-Zaguilan, R., & For-gac, M. (2015). Activity of plasma membrane V-ATPases is critical for the invasion of MDA-MB231 breast cancer cells. *The Journal of biological chemistry*, 290(6), 3680–3692. doi: 10.1074/jbc.M114.611210. <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4319033/>
- [7] Jiayu Gong, Chaoqian Cai, Xiaofeng Liu, Xin Ku, Hualiang Jiang, Daqi Gao and Honglin Li. ChemMapper: a Versatile Web Server for Exploring Pharmacology and Chemical Structure Association Based on Molecular 3D Similarity Method. *Bioinformatics*. 2013, 29 (14), 1827-1829. <https://pubmed.ncbi.nlm.nih.gov/23712658/>
- [8] Xiaofeng Liu, Hualiang Jiang, Honglin Li. SHAFTS: A Hybrid Approach for 3D Molecular Similarity Calculation. 1. Method and Assessment of Virtual Screening. *J. Chem. Inf. Model*. 2011, 51 (9), 2372-2385. <https://pubs.acs.org/doi/abs/10.1021/ci200060s>
- [9] Weiqiang Lu, Xiaofeng Liu, Xianwen Cao, Mengzhu Xue, Kangdong Liu, Zhenjiang Zhao, Xu Shen, Hualiang Jiang, Yufang Xu, Jin Huang and Honglin Li. SHAFTS: A Hybrid Approach for 3D Molecular Similarity Calculation. 2. Prospective Case Study in the Discovery of Diverse p90 Ribosomal S6 Protein Kinase 2 Inhibitors to Suppress Cell Migration. *J. Med. Chem*. 2011, 54, 3564-3574. <https://pubs.acs.org/doi/10.1021/jm200139j>
- [10] Calinescu AA, Kauss MC, Sultan Z, Al-Holou WN, O'Shea SK. Stem cells for the treatment of glioblastoma: a 20-year perspective. *CNS Oncol*. 2021 Jun 1;10(2):CNS73. doi: 10.2217/cns-2020-0026. Epub 2021 May 19. PMID: 34006134; PMCID: PMC8162173. <https://pubmed.ncbi.nlm.nih.gov/34006134/>
- [11] BioChemica. (2022). AEBSF hydrochloride BioChemica. PanReac AppliChem ITW Reagents. Retrieved from <https://www.itwreagents.com/italy/en/product/aebsf+hydrochloride+biochemica/A1421>
- [12] Nakabo Y, Pabst MJ. Lysis of leukemic cells by human macrophages: inhibition by 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF), a serine protease inhibitor. *J Leukoc Biol*. 1996 Sep;60(3):328-36. doi: 10.1002/jlb.60.3.328. PMID: 8830789. <https://pubmed.ncbi.nlm.nih.gov/8830789/>
- [13] Sreelatha L, Malakar S, Songprakhon P, Morchang A, Srisawat C, Noisakran S, Yenchi-tosomanus PT, Limjindaporn T. Serine protease inhibitor AEBSF reduces dengue virus infection via decreased cholesterol synthesis. *Virus Res*. 2019 Oct 2;271:197672. doi: 10.1016/j.virusres.2019.197672. Epub 2019 Aug 3. PMID: 31386864. <https://pubmed.ncbi.nlm.nih.gov/31386864/>
- [14] François Jouret, Céline Auzanneau, Huguette Debaix, Ge-Hong Sun Wada, Chrystel Pretto, Etienne Marbaix, Fiona E. Karet, Pierre J. Courtoy, Olivier Devuyst *JASN* Nov 2005, 16 (11) 3235-3246; DOI: 10.1681/ASN.2004110935. [https://jasn.asnjournals.org/content/16/11/3235#:~:text=The%20vacuolar%20H%2B%2DATPase%20\(of%20intercalated%20cells%20\(IC\).](https://jasn.asnjournals.org/content/16/11/3235#:~:text=The%20vacuolar%20H%2B%2DATPase%20(of%20intercalated%20cells%20(IC).)
- [15] Sarkar, J., Wen, X., Simanian, E. J., & Paine, M. L. (2016). V-type ATPase proton pump expression during enamel formation. *Matrix biology: journal of the International Society for Matrix Biology*, 52-54, 234–245. <https://doi.org/10.1016/j.matbio.2015.11.004>

## Cellular and Biomaterial Approaches for Treating Age-Related Macular Degeneration

Ashrit Challa\*, Srikar Sama, Selik Morrishetty

University of Pennsylvania, Philadelphia PA

**KEYWORDS:** Age-Related Macular Degeneration, Bioplastics, Tissue Engineering

**ABSTRACT:** Tissue Engineering offers a novel, curative approach to treating Age-Related Macular degeneration (AMD), a disease characterized by excessive drusen deposition beneath the retinal surface and consequent vision loss. Preclinical studies in rats have shown that transplanted Retinal Pigment Epithelium (RPE) derived from human Embryonic Stem Cells (hESC) have not only slowed AMD but have also restored vision. There are two main methods of delivering RPE cells: direct injection and monolayer surgical insertion, the latter demonstrating long-term integration. Biocompatible scaffolds allow for better delivery of RPE cells, induced Pluripotent Stem Cells (iPSC), and Retinal Progenitor Cells (RPC). Unlike animal-derived extracellular matrix components, soft modulus biomaterials such as poly(lactic-co-glycolic acid) (PLGA) and poly(l-lactic acid) (PLLA) are ideal for AMD cell transplants because of fast degradation times, high cellular attachment proliferation, and strong adherence to Bruch's membrane. These biomaterials can also be created at a 10-100 $\mu$ m thickness so that vision is not distorted. Use of biomaterials could be improved by cross-linking them with anti-vascular endothelial growth factors (VEGFs) like Brolucizumab and retinal growth factors such as fibroblast growth factor (FGF). Similarly, hESC and iPSC cells can be genetically modified to secrete anti-VEGF factors.

### INTRODUCTION

Age-related macular degeneration (AMD) is the leading cause of irreparable blindness in the developed world, affecting approximately 170 million people worldwide. Over 11 million people in the United States suffer from AMD, with that number projected to reach over 22 million over the coming decades [1]. People affected by AMD experience a significant decrease in their quality of life due to impaired visual acuity at or near 20/200 (Figure 1) [2].

While methods such as complement therapy and neuroprotection are currently being researched, there are no efficacious treatments for dry AMD. AMD must progress into the later and more severe wet AMD stage for current treatments to be effective [3]. The prevailing therapies of photodynamic therapy and anti-vascular endothelial growth factor (VEGF) therapy are inadequate, as their focus is not on curing AMD and restoring sight, but on slowing down and preventing further vision loss [4].

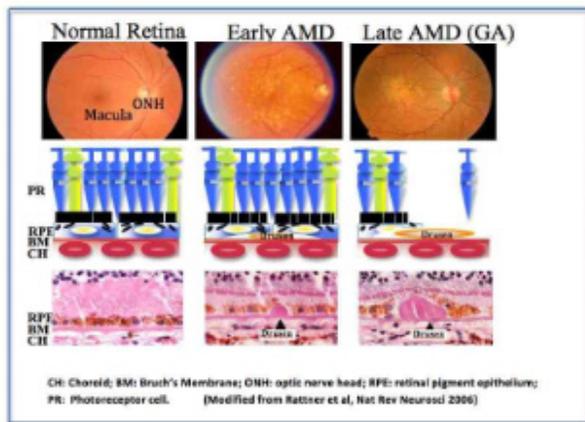


Figure 1. As AMD progresses, greater amounts of drusen disrupt the RPE-BM interface which leads to increased photoreceptor death.

As such, a tissue-engineered approach for treating AMD has impressive implications, potentially allowing for the reversal of a disease previously thought to be irreversible. This article reviews the clinical features of AMD, its current treatment options, stem cell and biomaterial tissue engineering therapeutic approaches, and pitfalls of and suggestions for such tissue-engineered approaches.

### CLINICAL FEATURES OF AMD

The clinical trademark of AMD is the accumulation of fat and protein deposits, commonly known as drusen, in the macula, an area that is dense with photoreceptors responsible for high visual acuity [5]. Drusen accumulates underneath the photoreceptors beneath the retinal pigment epithelium (RPE), which functions as a source of nutrients and growth factors, as well as a photoreceptor phagocytosis mechanism. The RPE attaches to Bruch's Membrane (BM), which acts as a barrier between the retina and the choroid while regulating diffusion between the choroid and the RPE [6]. While the disease can be categorized into early, interme-

diate, and late stages based on the extent of drusen proliferation and vision loss, the most important distinction remains between dry and wet AMD. Dry, or non-exudative, AMD occurs when excess drusen is deposited between the RPE and BM. This causes gradual RPE and photoreceptor cell death, as well as central macular atrophy and blind spots. Wet, or neovascular/exudative, AMD usually follows dry AMD and is characterized by choroidal neovascularization (CNV), or the invasion of choroidal blood vessels into the RPE. Bleeding and leaking from these vessels result in RPE cell death and rapid progression of blurriness and loss of visual acuity [7].

### CURRENT TREATMENTS AND MEDICATIONS FOR AMD

There are currently no effective curative treatments for AMD; however, existing therapies aim to manage the disease and stop its progression. Treatments for Wet AMD include laser photocoagulation and anti-VEGF therapy, such as Brolucizumab or Ranibizumab. Inhibiting vascularization in the eye prevents further progress of CNV, but it also contributes to chorioretinal atrophy due to less vascularization and potential narrowing of choroid capillaries. In fact, followups with anti-VEGF treated eyes indicate an extremely high level (98%) of macular atrophy, particularly in the fovea [8]. Photodynamic therapy (PDT) like Verteporfin aims to simply stop the progression of destructive vascularization through laser-activated medication. However, there is no clinically significant improvement of visual acuity because of this therapeutic method [9]. As such, monthly anti-VEGF drug injections are currently prescribed and have had minimal to moderate success in restoring some vision to patients (about 30% of treated individuals), only maintaining the eye equivalent to its initial

state in which treatment first began. Even when PDT and anti-VEGF drugs are used in conjunction, a secondary approach when neither therapy is individually effective, there is a similar rate of visual acuity improvement (~11-13 letters) [10]. Recent advancements in pharmacology have led to the development of better-performing injections, such as Brolucizumab, which has a higher rate of visual acuity correction than Ranibizumab. Coupled with its small size, this compound allows for better vision improvement with fewer injections, but still holds the risk of injection-related complications and excessive drying [11]. Currently, there are no current treatments for dry AMD besides a surgical transplantation of a homologous donor retina. Transplantation has previously been shown to be ineffective due to a failure in synapse formation between fully differentiated tissue and the host. Surgical approaches of cleaning the debris near the retina and attempting to replace the degenerating retina with bolus injections have only offered temporary respite to the afflicted retina as it regresses back to its damaged form [12].

### CELL THERAPY APPROACHES

Due to the difficulties of conventional therapies, recent years have witnessed much research and development in the use of stem cells and induced pluripotent cells as treatment or cures for AMD. In 1987, the first significant study on the use of transplanted RPE cells for treatment was conducted by Gouras et al, who placed transplanted rabbit RPE adjacent to the neural retina of a different rabbit [13]. Another landmark occurred ten years later, when Al-gverre et al. transplanted human fetal RPE patches into the subretinal space of human patients with wet or dry AMD. The results of this grafting indicated that RPE transplants would not be rejected and further degrade vision,

even without immunosuppression, and that dry AMD had a lower graft rejection rate [14]. Since these landmark studies of RPE transplants, iris pigment epithelium (IPE) cells and human embryonic stem cells (hESC) have been transplanted in many animal models, showing vision improvement. Additionally, researchers have transplanted RPE cells, choroid-Bruch's-RPE explants, IPE cells and hESC in human patients with AMD, aiming to evaluate transplantation safety (Figure 2) [15].

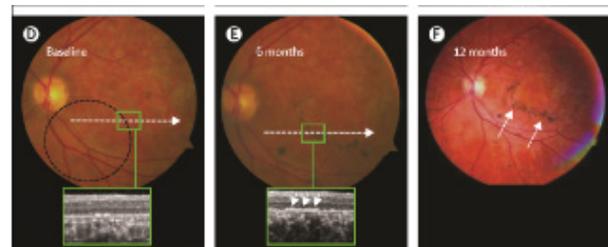


Figure 2: hESC-RPE cells injected into the subretinal space of human patients exhibit increased pigmentation and result in regeneration of the macula.

Preclinical studies conducted in rats have shown that using transplanted RPE cells derived from hESC have slowed retinal degeneration [16] and even improved visual acuity [17][18]. Additionally, hESC-RPE has formed a polarized epithelial layer in vitro, secreting growth factors such as pigment epithelium described factor and VEGF, all while expressing the barrier properties of normal adult human RPE cells [19]. Currently the two preferred methods for delivering hESC-RPE into the subretinal space are either injections of cells suspended in a fluid, which are inexpensive and simple but carry the risk of RPE cell dedifferentiation, or creating monolayers of hESC-RPE which can be surgically placed subretinally, necessitating a biologically compatible substrate [20]. Monolayers have shown higher rates of cell survival in comparison to injected

cell suspensions, as well as less clumping of cells [21]. Recent advancements with hESC-RPE therapy include a completed three-year and an ongoing clinical trial conducted in part by the London Project to Cure Blindness, with the former demonstrating the safety of long-term grafts while being the first to record longitudinal effects of hESC-RPE monolayer implantations. The completed study also showed lasting improvement in visual acuity of about 14-15 letters, as well as no adverse proliferative reactions such as teratoma formation, even after 37 months of observation. Similarly, the phase I results of the ongoing clinical trial, with interventions provided to two human patients, indicate hESC-RPE integration while presenting improvement of visual acuity and reading speeds [22][23]. While the former study was conducted using bolus cell injections of hESC-RPEs, the latter utilized a polyester sub-strate for monolayer insertion. Furthermore, there are also other clinical trials using cell sheets, such as a trial using induced pluripotent stem cell (iPSC)-RPE cell sheet transplantation in patients with dry AMD rather than wet AMD, although this trial has only very recently begun (NCT04339764). Though the retinal space in the eye is immunologically privileged, various studies have used immunosuppression (such as tacrolimus and mycophenolate mofetil) during their trials of implanted stem cells, while others have relied purely on the immunological status of the eye [24]. Both methods have had been successful in avoiding immunological rejection.

iPSCs have been studied for in vivo curative treatments and for the modeling of AMD and other eye-related diseases. This is because there is an information deficit in the exact mechanisms for the progression of AMD, specifically the non-exudative form. In response, the wide range of iPSC potential differentiation offers novel breakthrough methods in terms of replace-

ment therapy and disease modeling [25]. To dedifferentiate human fibroblasts for use in iPSC-RPE procedures and other iPSC techniques, cells can be transfected with vectors such as the Venezuelan Equine Encephalitis RNA vector, inducing exogenous expression of pluripotency markers such as OCT4, SOX2, KLF4, and GLIS1. With a dedifferentiation rate of >95%, newly formed iPSC cells are then suspended to form embryoid bodies, which are then placed in RPE medium. This allows for iPSC-RPE cells from different lineages to express high levels of RPE genes and proteins, such as RPE65 and MERTK [26]. Already, iPSC-RPE cells have been used to better understand the molecular etiology of AMD, with one study identifying a single-nucleotide polymorphism near the VEGFA gene in AMD patients that decreases gene expression (Figure 3). In conjunction, another study has been able to identify genes that are differently regulated in AMD patients, alongside cell proliferation and localized immune response changes [27][28]. In this vein, iPSC-RPE research is exciting in its ability to better elucidate previously unknown mechanisms in AMD development.

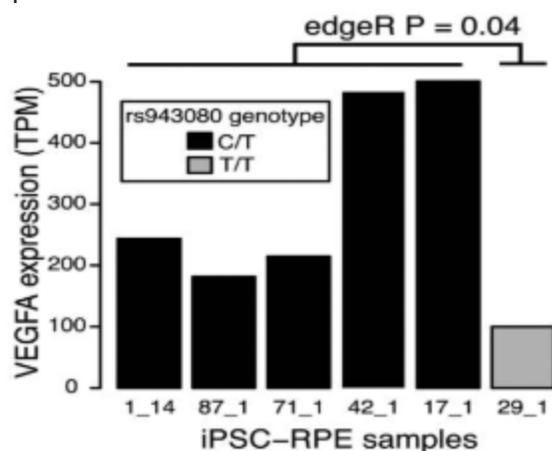


Figure 3. Expression levels of VEGFA for six iPSC-RPE samples, with the risk variant/ AMD SNP sample exhibiting significantly lower amounts of the gene

### BIOMATERIALS APPROACHES

Surgeons currently do not have complete control over where the RPE or retinal progenitor cells (RPCs) are placed subretinally, with random clumps of cells not being conducive to regeneration. Additionally, without a way to ascertain the polarity and orientation of injected bolus or unoriented sheet cells, adherence to Bruch's membrane is drastically lowered. The use of biocompatible materials allows the parameters for delivery to be adjusted so that RPE cells, iPSCs, and other RPCs can be delivered subretinally to regenerate the damaged RPE cell layer, attaching to photoreceptors apically and BM basally. Thus, biomaterials must allow for cell attachment, proliferation, and correct orientation/polarization. To allow for RPE cell proliferation and BM attachment, the material must degrade by 2-3 weeks post-implant, and is ideally 10-100 $\mu$ m to allow for precise manipulation without retinal distortion and nutrient diffusion limitation [29].

Some biomaterials that have been explored as substrates for RPE monolayer insertion are collagen, Matrigel®, fibronectin, laminin, vitronectin, and oligopeptides. However, these organic, animal-derived extracellular matrix components were found to discourage cell proliferation and have variable degradation times based on individual enzymatic digestion rates [30]. Nanowires of poly( $\epsilon$ -caprolactone) (PCL) may also be cast, but require precise construction in order to ensure porosity levels conducive to RPC polarization and attachment [31]. Poly(DL-lactic-co-glycolic acid) (PLGA), poly(glycolic acid) (PGA), poly(ethylene glycol) (PEG), and poly(DL-lactic acid) (PLA), are synthetic, thin, and degradable bio-materials. However, PLGA has an ideal degradation time of 2-3 weeks (whereas the others do not significantly degrade until about four weeks after initial cell seeding), can be 10-130 $\mu$ m thick,

and has a feasible manufacturing process [32] [33]. Once solvated in chloroform or hexafluoroisopropanol (HFIP), these polymers are left to deposit on an even glass or Scaffdex surface for 8 hours while the solvent evaporates. The thin sheets are then left to dry, potentially stored in nitrogenous atmospheres over desiccators like calcium sulfate. They may then be crosslinked and sterilized using UV light [34][35]. The RPE, RPC, and iPSCs can thus be seeded. It has been found that there is 99-100% attachment of non-hESC human RPE cells to PLGA after 8 hours, and both PLGA and PGA sheets allow for RPE cell metabolism and protein expression. These polymers are therefore viable for cell attachment and proliferation, allowing for apical microvilli and basilar diffusion and molecule excretion [36][37]. However, these RPE cells do not attach significantly to PLA or PEG. After 3-7 days, cells become confluent, forming a multi-layer or monolayer (depending on the cell type) of polarized RPE or RPCs and are ready for implantation.

PLGA sheets have been found to be the smoothest, thinnest, have the highest polarized RPE cell attachment, and most proliferation/material area repopulation. This is due to a 50:50 high molecular weight PGLA that contains an optimal ratio of lactic acid to glycolic acid. In comparison to previous substrates such as collagen, PLGA sheets are much smoother, thinner, and allow for the formation of an RPE monolayer with both correct orientation and polarity of cells. Additionally, multiple PLGA ratios of lactic acid to glycolic acid, such as high molecular weight (high MW) 50:50 and 75:25 PLGA, allowed for significant levels of cell proliferation. However, due to its faster degradation time, a high MW 50:50 PLGA blend is thought to be optimal [38]. The porosity of this blend allows correct adherence to BM so that as the biomaterial is degraded, the RPE cells attach

and are integrated into the eye. Studies also indicate that creating a polymer blend of PLGA and poly(L-lactic acid) (PLLA) may improve the porosity of the substrate, thus allowing for higher rates of cell proliferation. The modulus (hardness) of PLGA and poly(L-lactic acid) PLLA 50:50 is low enough to overcome the otherwise stiff composition of pure PLGA, with the flexibility provided by PLLA mitigating the risk of retinal damage. This 50:50 PLGA:PLLA was implanted in the rat model and the viability of the cells was monitored for 14 days [39]. Unlike previous attempts to directly inject RPC cells into the retina, the PLGA:PLLA bound RPC and RPE cells were still viable and expressed GFP, unlike transplanted cells, which only had a 10% survival rate 14 days after implantation (Figure 4). A more recent study shows that a 25:75 PLLA:PLGA blend might be more efficient, with a higher level of porosity and a lower elastic modulus (Figure 5) [40]. Soft modulus biomaterials of PLGA and PLLA are ideal for AMD cell delivery because they degrade fast, have high cell attachment, RPE and RPC cell polarization, cell proliferation, adherence to BM, and high viability after implantation.

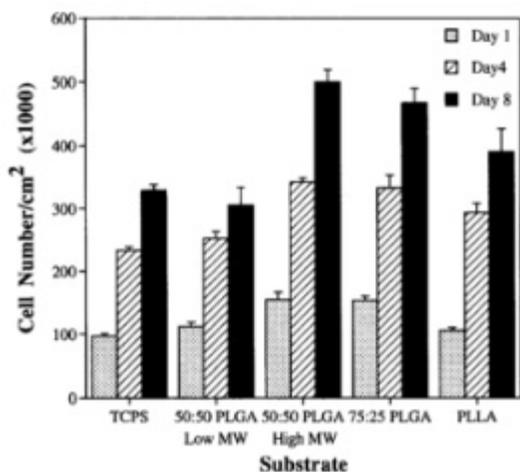


Figure 4. Attachment levels of RPE cells for different cell substrates, with three different compositions of PLGA, the most proliferative substrate

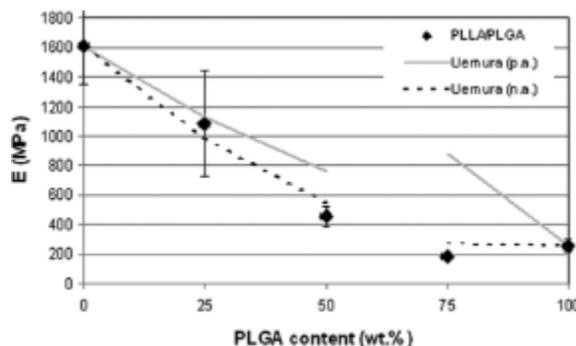


Figure 5: Predicted vs. Actual elastic moduli for various PLLA: PLGA ratios, with the 25:75 exhibiting the lowest modulus

### SHORTCOMINGS

Although cell therapies have evolved greatly over the past few decades, regarding both transplantations and surgical monolayer integration, there are still many unanswered questions. Transplanted hESC-RPE cells demonstrated an increase in retinal pigmentation and visual acuity by about 14 letters in patients [41]. However, a 2008 study that surgically implanted fetal RPE into ten patients saw a four times improvement in visual acuity in one patient, from 20/800 to 20/200, which remained stable for five years [42]. This magnitude in visual improvement has not yet been seen from hESC or iPSC approaches, which are also marred by complications such as cataract formation and vitreous inflammation. And while iPSC-RPE clinical trial results are increasing in number, there is no published research on the longitudinal effects of iPSC-RPE cell transplantation in human patients with AMD. It is known that iPSC-RPE cells also have a faster rejection time than hESC-derived cells, triggering macrophage-mediated phagocytosis such that almost no iPSC cells remain after 13 weeks [43].

Given that donor retina scaffolds are difficult to procure, tissue engineering approaches provide alternative avenues. Regardless, the

use of cutting-edge stem cell engineering techniques and substrates like PLGA and PLLA still pose problems. Some of these problems include inflammation and injury due to the injection of cells, incomplete attachment of RPE cells to BM or a lack of subsequent proliferation, and the inability to replicate the true retinal environment. Cells may also dedifferentiate once attached to BM, which would only lead to the presence of more harmful debris in the retina and subretinal space. Both injected and surgically inserted cells have not yet shown complete restorations of vision and have only demonstrated peripheral and minor macular vision improvement. Finally, the toxicity of the material used as the cell substrate must also be considered, as degradation of these scaffolds will inevitably lead to the presence of small subunits in the macular region. With the use of PLGA, a potentially toxic and immunogenic substance in the eye, careful construction of scaffolding must be used in accordance with shape and size restraints. As more data comes out regarding biomaterial interactions with the actual human eye, rather than approximating animal models, it will be important to modify scaffold compositions and morphologies accordingly [44].

### **IMPROVEMENTS FOR CURRENT TECHNIQUES**

Based on current research, there are many hypothesized methods of improving the treatment of AMD. Cell therapies utilizing stem cells can be further improved with genetically modified RPE cells, able to impede or revert the progression of AMD through genome and transcriptome modifications to counteract the changes of the disease. Neovascular AMD is due in part to an imbalance in growth factors such as VEGF. RPE cells naturally express many anti-VEGFs like pigment epithelium-derived factor (PEDF), but damage to these cells allows for ex-

cess vascularization. To further treatment, transplanted cells should be genetically modified to upregulate the production of anti-VEGFs, such as endostatin, PEDF, basic fibroblast growth factor (bFGF), brain-derived neurotrophic factor (BDNF), or ciliary neurotrophic factor (CNTF). To perform the necessary genetic modifications for the aforementioned suggestions, possible transfection methods include the Sleeping Beauty (SB100X)<sup>^</sup> transposon system or the CRISPR/Cas9 system. During the later stages of AMD when damage has occurred to both photoreceptors and RPE cells, another improvement would be to transplant autologous ESC or iPSC which differentiate under a wider range of factors. This would allow for the reconstruction of the entire BM-RPE-photoreceptor complex, potentially leading to greater improvements in vision. In addition to therapeutic uses, it would also be helpful to use iPSC-RPE cells to model dry AMD alongside wet AMD in humans, as these cells could uncover more about dry AMD while finding use as high throughput drug screens. Gene therapy efficacies could also be tested in this way. This knowledge could lead to not just curative methods, but perhaps preventative interventions as well.

In using biomaterials as a substrate for RPE cells, endogenous factors can perhaps be crosslinked to PLGA or PLLA. As proteases break the crosslinks in PLGA scaffolds, anti-VEGFs such as Ranibizumab and retinal growth factors like FGF could be freed, allowing for retinal regeneration [45]. Delivery of Ranibizumab via nanoparticles has already been demonstrated, so it is now a matter of delivering these types of medications on PLGA scaffolds for AMD patients (Figure 6) [46].

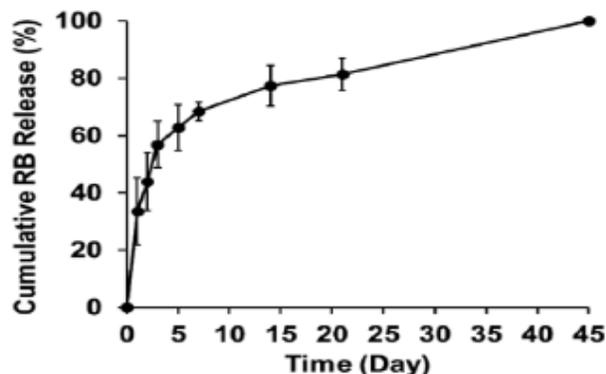


Figure 6: Cumulative Release percentage of Ranibizumab by PLGA nanoparticle system; if modified into PLGA scaffolding, could be used to deliver anti-VEGFs to integrating RPE cells

Because RPE cells must be polarized, incorporating PLGA with this cell type alongside RPCs and iPSCs may allow for differentiation into RPE and other retinal cells. In this manner, the eye can heal any damage done by the degradation or delivery of the substrate biomaterial and cells in the first place. Similarly, the use of drugs to modulate calcium systems has shown down-regulating effects on proliferation, which could be used in the case of teratoma creating iPSC-RPE cells. If cross-linked in PLGA scaffolds, these inhibitors could be used as a form of cell control [47]. In regard to surgical placement, current methods require the rolling up of PLGA/PLLA scaffolds. These rolled-up scaffolds are then delivered to the subretinal space. An improvement would be to develop a method by which a higher modulus material can be delivered to the eye directly in between the BM and the photoreceptors, perhaps via a small incision. This higher modulus material could then be removed to leave softer biomaterials, which are less harmful to retinal health and vision. Finally, cleaning up dedifferentiated cell and degraded scaffolding debris with lasers may also allow for better vision and healing.

## CONCLUSION

Age-related macular degeneration is one of the leading causes of blindness in aging adults and is prevalent in today's population. Tissue engineering solutions for AMD have improved upon conventional treatments, thanks to their ability to not just stop the progression of AMD, but also result in visual improvement. The use of hESC-RPE and iPSC-RPE, coupled with a thin PLGA/PLLA scaffold, may allow for effective and safe integration of RPE cells in the macula. With more research on polymer toxicity, molecular crosslinking, and methods of efficient insertion, these cellular engineering techniques may become the best way to reverse AMD damage and fully regenerate the patient's eye.

## AUTHOR INFORMATION

### Corresponding Author

\*Ashrit Challa, University of Pennsylvania, Department of Biology; achalla@sas.upenn.edu, 937-750-1530

### Author Contributions

Ashrit Challa – Text  
Srikar Sama – Figures and Proofreading  
Selik Morishetty – Figures and Proofreading

## ABBREVIATIONS

AMD – Age-Related Macular Degeneration  
VEGF – Vascular Endothelial Growth Factor  
RPE – Retinal Pigment Epithelium  
BM – Bruch's Membrane  
CNV – Choroidal Neovascularization  
PDT – Photodynamic Therapy  
IPE – Iris Pigment Epithelium  
hESC – Human Embryonic Stem Cells  
iPSC – Induced Pluripotent Stem Cells  
RPC – Retinal Progenitor Cell  
PLGA – Poly(dl-lactic-co-glycolic acid)  
PGA – Poly(glycolic acid)

PEG – Poly(ethylene glycol)  
PLA – Poly(dl-lactic acid)  
HFIP - Hexafluoroisopropanol  
PLLA – Poly(l-lactic acid)  
PEDF – Pigment epithelium-derived factor  
bFGF – Basic fibroblast growth factor  
BDNF – Brain-derived neurotrophic factor  
CNTF – Ciliary neurotrophic factor

## REFERENCES

- [1] Pennington, K. L., & DeAngelis, M. M. (2016). Epidemiology of age-related macular degeneration (AMD): associations with cardiovascular disease phenotypes and lipid factors. *Eye and vision (London, England)*, 3, 34. <https://doi.org/10.1186/s40662-016-0063-5>
- [2] Fernández-Robredo, P., Sancho, A., Johnen, S., Recalde, S., Gama, N., Thumann, G., Groll, J., & García-Layana, A. (2014). Current treatment limitations in age-related macular degeneration and future approaches based on cell therapy and tissue engineering. *Journal of ophthalmology*, 2014, 510285. <https://doi.org/10.1155/2014/510285>
- [3] Ammar, M. J., Hsu, J., Chiang, A., Ho, A. C., & Regillo, C. D. (2020). Age-related macular degeneration therapy: a review. *Current opinion in ophthalmology*, 31(3), 215–221. <https://doi.org/10.1097/ICU.0000000000000657>
- [4] Fernández-Robredo, P., Sancho, A., Johnen, S., Recalde, S., Gama, N., Thumann, G., Groll, J., & García-Layana, A. (2014). Current treatment limitations in age-related macular degeneration and future approaches based on cell therapy and tissue engineering. *Journal of ophthalmology*, 2014, 510285. <https://doi.org/10.1155/2014/510285>
- [5] Jager, R. D., Mieler, W. F., & Miller, J. W. (2008). Age-related macular degeneration. *The New England journal of medicine*, 358(24), 2606–2617. <https://doi.org/10.1056/NEJM-ra0801537>
- [6] Bhutto, I., & Lutty, G. (2012). Understanding age-related macular degeneration (AMD): relationships between the photoreceptor/retinal pigment epithelium/Bruch's membrane/choriocapillaris complex. *Molecular aspects of medicine*, 33(4), 295–317. <https://doi.org/10.1016/j.mam.2012.04.005>
- [7] Mathenge W. (2014). Age-related macular degeneration. *Community eye health*, 27(87), 49–50.
- [8] Fernández-Robredo, P., Sancho, A., Johnen, S., Recalde, S., Gama, N., Thumann, G., Groll, J., & García-Layana, A. (2014). Current treatment limitations in age-related macular degeneration and future approaches based on cell therapy and tissue engineering. *Journal of ophthalmology*, 2014, 510285. <https://doi.org/10.1155/2014/510285>
- [9] Brown, D. M., Kaiser, P. K., Michels, M., Soubrane, G., Heier, J. S., Kim, R. Y., Sy, J. P., Schneider, S., & ANCHOR Study Group (2006). Ranibizumab versus verteporfin for neovascular age-related macular degeneration. *The New England journal of medicine*, 355(14), 1432–1444. <https://doi.org/10.1056/NEJMoa062655>
- [10] Fernández-Robredo, P., Sancho, A., Johnen, S., Recalde, S., Gama, N., Thumann, G., Groll, J., & García-Layana, A. (2014). Current treatment limitations in age-related macular degeneration and future approaches based on cell therapy and tissue engineering. *Journal of ophthalmology*, 2014, 510285. <https://doi.org/10.1155/2014/510285>

- [11] Yannuzzi, N. A., & Freund, K. B. (2019). Brolucizumab: evidence to date in the treatment of neovascular age-related macular degeneration. *Clinical ophthalmology (Auckland, N.Z.)*, 13, 1323–1329. <https://doi.org/10.2147/OPHTH.S184706>
- [12] MacLaren, R. E., Bird, A. C., Sathia, P. J., & Aylward, G. W. (2005). Long-term results of submacular surgery combined with macular translocation of the retinal pigment epithelium in neovascular age-related macular degeneration. *Ophthalmology*, 112(12), 2081–2087. <https://doi.org/10.1016/j.optha.2005.06.029>
- [13] Lopez, R., Gouras, P., Brittis, M., & Kjeldbye, H. (1987). Transplantation of cultured rabbit retinal epithelium to rabbit retina using a closed-eye method. *Investigative ophthalmology & visual science*, 28(7), 1131–1137.
- [14] Algvere, P. V., Berglin, L., Gouras, P., Sheng, Y., & Kopp, E. D. (1997). Transplantation of RPE in age-related macular degeneration: observations in disciform lesions and dry RPE atrophy. *Graefes archive for clinical and experimental ophthalmology = Albrecht von Graefes Archiv fur klinische und experimentelle Ophthalmologie*, 235(3), 149–158. <https://doi.org/10.1007/BF00941722>
- [15] Fernández-Robredo, P., Sancho, A., Johnen, S., Recalde, S., Gama, N., Thumann, G., Groll, J., & García-Layana, A. (2014). Current treatment limitations in age-related macular degeneration and future approaches based on cell therapy and tissue engineering. *Journal of ophthalmology*, 2014, 510285. <https://doi.org/10.1155/2014/510285>
- [16] Idelson, M., Alper, R., Obolensky, A., Ben-Shushan, E., Hemo, I., Yachimovich-Cohen, N., Khaner, H., Smith, Y., Wiser, O., Gropp, M., Cohen, M. A., Even-Ram, S., Berman-Zaken, Y., Matzrafi, L., Rechavi, G., Banin, E., & Reubinoff, B. (2009). Directed differentiation of human embryonic stem cells into functional retinal pigment epithelium cells. *Cell stem cell*, 5(4), 396–408. <https://doi.org/10.1016/j.stem.2009.07.002>
- [17] Lu, B., Malcuit, C., Wang, S., Girman, S., Francis, P., Lemieux, L., Lanza, R., & Lund, R. (2009). Long-term safety and function of RPE from human embryonic stem cells in preclinical models of macular degeneration. *Stem cells (Dayton, Ohio)*, 27(9), 2126–2135. <https://doi.org/10.1002/stem.149>
- [18] Lund, R. D., Wang, S., Klimanskaya, I., Holmes, T., Ramos-Kelsey, R., Lu, B., Girman, S., Bischoff, N., Sauv e, Y., & Lanza, R. (2006). Human embryonic stem cell-derived cells rescue visual function in dystrophic RCS rats. *Cloning and stem cells*, 8(3), 189–199. <https://doi.org/10.1089/clo.2006.8.189>
- [19] Carr, A. J., Smart, M. J., Ramsden, C. M., Powner, M. B., da Cruz, L., & Coffey, P. J. (2013). Development of human embryonic stem cell therapies for age-related macular degeneration. *Trends in neurosciences*, 36(7), 385–395. <https://doi.org/10.1016/j.tins.2013.03.006>
- [20] Carr, A. J., Smart, M. J., Ramsden, C. M., Powner, M. B., da Cruz, L., & Coffey, P. J. (2013). Development of human embryonic stem cell therapies for age-related macular degeneration. *Trends in neurosciences*, 36(7), 385–395. <https://doi.org/10.1016/j.tins.2013.03.006>
- [21] Diniz, B., Thomas, P., Thomas, B., Ribeiro, R., Hu, Y., Brant, R., Ahuja, A., Zhu, D., Liu, L., Koss, M., Maia, M., Chader, G., Hinton, D. R., & Humayun, M. S. (2013). Subretinal implantation

of retinal pigment epithelial cells derived from human embryonic stem cells: improved survival when implanted as a monolayer. *Investigative ophthalmology & visual science*, 54(7), 5087–5096. <https://doi.org/10.1167/iovs.12-11239>

[22] Schwartz, S. D., Regillo, C. D., Lam, B. L., Elliott, D., Rosenfeld, P. J., Gregori, N. Z., Hubschman, J. P., Davis, J. L., Heilwell, G., Sporn, M., Maguire, J., Gay, R., Bateman, J., Ostrick, R. M., Morris, D., Vincent, M., Anglade, E., Del Priore, L. V., & Lanza, R. (2015). Human embryonic stem cell-derived retinal pigment epithelium in patients with age-related macular degeneration and Stargardt's macular dystrophy: follow-up of two open-label phase 1/2 studies. *Lancet (London, England)*, 385(9967), 509–516. [https://doi.org/10.1016/S0140-6736\(14\)61376-3](https://doi.org/10.1016/S0140-6736(14)61376-3)

[23] da Cruz, L., Fynes, K., Georgiadis, O., Kerby, J., Luo, Y. H., Ahmado, A., Vernon, A., Daniels, J. T., Nommiste, B., Hasan, S. M., Gooljar, S. B., Carr, A. F., Vugler, A., Ramsden, C. M., Bictash, M., Fenster, M., Steer, J., Harbinson, T., Wilbrey, A., Tufail, A., ... Coffey, P. J. (2018). Phase 1 clinical study of an embryonic stem cell-derived retinal pigment epithelium patch in age-related macular degeneration. *Nature biotechnology*, 36(4), 328–337. <https://doi.org/10.1038/nbt.4114>

[24] Lu, B., Malcuit, C., Wang, S., Girman, S., Francis, P., Lemieux, L., Lanza, R., & Lund, R. (2009). Long-term safety and function of RPE from human embryonic stem cells in preclinical models of macular degeneration. *Stem cells (Dayton, Ohio)*, 27(9), 2126–2135. <https://doi.org/10.1002/stem.149>

[25] Melville, H., Carpiello, M., Hollis, K., Staffaroni, A., & Golestanian, N. (2013). Stem cells: a new paradigm for disease modeling and

developing therapies for age-related macular degeneration. *Journal of translational medicine*, 11, 53. <https://doi.org/10.1186/1479-5876-11-53>

[26] Hazim, R. A., Karumbayaram, S., Jiang, M., Dimashkie, A., Lopes, V. S., Li, D., Burgess, B. L., Vijayaraj, P., Alva-Ornelas, J. A., Zack, J. A., Kohn, D. B., Gomperts, B. N., Pyle, A. D., Lowry, W. E., & Williams, D. S. (2017). Differentiation of RPE cells from integration-free iPS cells and their cell biological characterization. *Stem cell research & therapy*, 8(1), 217. <https://doi.org/10.1186/s13287-017-0652-9>

[27] Smith, E. N., D'Antonio-Chronowska, A., Greenwald, W. W., Borja, V., Aguiar, L. R., Pogue, R., Matsui, H., Benaglio, P., Bo-rooah, S., D'Antonio, M., Ayyagari, R., & Frazer, K. A. (2019). Human iPSC-Derived Retinal Pigment Epithelium: A Model System for Prioritizing and Functionally Characterizing Causal Variants at AMD Risk Loci. *Stem cell reports*, 12(6), 1342–1353. <https://doi.org/10.1016/j.stemcr.2019.04.012>

[28] Gong, J., Cai, H., NYSCF Global Stem Cell Array Team, Noggle, S., Paull, D., Rizzolo, L. J., Del Priore, L. V., & Fields, M. A. (2020). Stem cell-derived retinal pigment epithelium from patients with age-related macular degeneration exhibit reduced metabolism and matrix interactions. *Stem cells translational medicine*, 9(3), 364–376. <https://doi.org/10.1002/sctm.19-0321>

[29] Lu, L., Yaszemski, M. J., & Mikos, A. G. (2001). Retinal pigment epithelium engineering using synthetic biodegradable polymers. *Biomaterials*, 22(24), 3345–3355. [https://doi.org/10.1016/s0142-9612\(01\)00172-7](https://doi.org/10.1016/s0142-9612(01)00172-7)

[30] Lu, L., Yaszemski, M. J., & Mikos, A. G. (2001). Retinal pigment epithelium engineer-

ing using synthetic biodegradable polymers. *Biomaterials*, 22(24), 3345–3355. [https://doi.org/10.1016/s0142-9612\(01\)00172-7](https://doi.org/10.1016/s0142-9612(01)00172-7)

[31] McHugh, K. J., Tao, S. L., & Saint-Geniez, M. (2014). Porous poly( $\epsilon$ -caprolactone) scaffolds for retinal pigment epithelium transplantation. *Investigative ophthalmology & visual science*, 55(3), 1754–1762. <https://doi.org/10.1167/iops.13-12833>

[32] Reed, A. M., Gilding, D. K. (1981). Biodegradable polymers for use in surgery-poly(glycolic)/poly(lactic acid) homo and copolymers: 2. In vitro degradation. *Polymer*, 22(4), 494-498. [https://doi.org/10.1016/0032-3861\(81\)90168-3](https://doi.org/10.1016/0032-3861(81)90168-3)

[33] McCormick, R., Pearce, I., Kaye, S., & Haneef, A. (2020). Optimisation of a Novel Bio-Substrate as a Treatment for Atrophic Age-Related Macular Degeneration. *Frontiers in bioengineering and bio-technology*, 8, 456. <https://doi.org/10.3389/fbioe.2020.00456>

[34] Lu, L., Nyalakonda, K., Kam, L., Bizios, R., Göpferich, A., & Mikos, A. G. (2001). Retinal pigment epithelial cell adhesion on novel micropatterned surfaces fabricated from synthetic biodegradable polymers. *Biomaterials*, 22(3), 291–297. [https://doi.org/10.1016/s0142-9612\(00\)00179-4](https://doi.org/10.1016/s0142-9612(00)00179-4)

[35] McCormick, R., Pearce, I., Kaye, S., & Haneef, A. (2020). Optimisation of a Novel Bio-Substrate as a Treatment for Atrophic Age-Related Macular Degeneration. *Frontiers in bioengineering and bio-technology*, 8, 456. <https://doi.org/10.3389/fbioe.2020.00456>

[36] Lu, L., Garcia, C. A., & Mikos, A. G. (1998). Retinal pigment epithelium cell culture on thin biodegradable poly(DL-lactic-co-glycolic acid)

films. *Journal of biomaterials science. Polymer edition*, 9(11), 1187–1205. <https://doi.org/10.1163/156856298x00721>

[37] McCormick, R., Pearce, I., Kaye, S., & Haneef, A. (2020). Optimisation of a Novel Bio-Substrate as a Treatment for Atrophic Age-Related Macular Degeneration. *Frontiers in bioengineering and bio-technology*, 8, 456. <https://doi.org/10.3389/fbioe.2020.00456>

[38] Giordano, G. G., Thomson, R. C., Ishaug, S. L., Mikos, A. G., Cumber, S., Garcia, C. A., & Lahiri-Munir, D. (1997). Retinal pigment epithelium cells cultured on synthetic biodegradable polymers. *Journal of biomedical materials research*, 34(1), 87–93. [https://doi.org/10.1002/\(sici\)1097-4636\(199701\)34:1<87::aid-jbm12>3.0.co;2-m](https://doi.org/10.1002/(sici)1097-4636(199701)34:1<87::aid-jbm12>3.0.co;2-m)

[39] Lavik, E. B., Klassen, H., Warfvinge, K., Langer, R., & Young, M. J. (2005). Fabrication of degradable polymer scaffolds to direct the integration and differentiation of retinal progenitors. *Biomaterials*, 26(16), 3187–3196. <https://doi.org/10.1016/j.biomaterials.2004.08.022>

[40] Thomson, H. A., Treharne, A. J., Walker, P., Gossel, M. C., & Lotery, A. J. (2011). Optimisation of polymer scaffolds for retinal pigment epithelium (RPE) cell transplantation. *The British journal of ophthalmology*, 95(4), 563–568. <https://doi.org/10.1136/bjo.2009.166728>

[41] Schwartz, S. D., Regillo, C. D., Lam, B. L., Elliott, D., Rosenfeld, P. J., Gregori, N. Z., Hubschman, J. P., Davis, J. L., Heilwell, G., Sporn, M., Maguire, J., Gay, R., Bateman, J., Ostrick, R. M., Morris, D., Vincent, M., Anglade, E., Del Priore, L. V., & Lanza, R. (2015). Human embryonic stem cell-derived retinal pigment epithelium in patients with age-related

macular degeneration and Stargardt's macular dystrophy: follow-up of two open-label phase 1/2 studies. *Lancet* (London, England), 385(9967), 509–516. [https://doi.org/10.1016/S0140-6736\(14\)61376-3](https://doi.org/10.1016/S0140-6736(14)61376-3)

[42] Radtke, N. D., Aramant, R. B., Petry, H. M., Green, P. T., Pidwell, D. J., & Seiler, M. J. (2008). Vision improvement in retinal degeneration patients by implantation of retina together with retinal pigment epithelium. *American journal of ophthalmology*, 146(2), 172–182. <https://doi.org/10.1016/j.ajo.2008.04.009>

[43] Carr, A. J., Smart, M. J., Ramsden, C. M., Powner, M. B., da Cruz, L., & Coffey, P. J. (2013). Development of human embryonic stem cell therapies for age-related macular degeneration. *Trends in neurosciences*, 36(7), 385–395. <https://doi.org/10.1016/j.tins.2013.03.006>

[44] Thackaberry, E. A., Farman, C., Zhong, F., Lorget, F., Staflin, K., Cercillieux, A., Miller, P. E., Schuetz, C., Chang, D., Famili, A., Daugherty, A. L., Rajagopal, K., & Bantseev, V. (2017). Evaluation of the Toxicity of Intravitreally Injected PLGA Microspheres and Rods in Monkeys and Rabbits: Effects of Depot Size on Inflammatory Response. *Investigative ophthalmology & visual science*, 58(10), 4274–4285. <https://doi.org/10.1167/iovs.16-21334>

[45] Sievers, J., Hausmann, B., Unsicker, K., Berry, M. (1987). Fibroblast growth factors promote the survival of adult rat retinal ganglion cells after transection of the optic nerve. *Neuroscience Letters*, 76(2), 157-162. [https://doi.org/10.1016/0304-3940\(87\)90708-7](https://doi.org/10.1016/0304-3940(87)90708-7)

[46] Tanetsugu, Y., Tagami, T., Terukina, T., Ogawa, T., Ohta, M., & Ozeki, T. (2017). Development of a Sustainable Release System for a

Ranibizumab Biosimilar Using Poly(lactic-co-glycolic acid) Biodegradable Polymer-Based Microparticles as a Platform. *Biological & pharmaceutical bulletin*, 40(2), 145–150. <https://doi.org/10.1248/bpb.b16-00437>

[47] Wagner, M., Benson, M. T., Rennie, I. G., & MacNeil, S. (1995). Effects of pharmacological modulation of intracellular signalling systems on retinal pigment epithelial cell attachment to extracellular matrix proteins. *Current eye research*, 14(5), 373–384. <https://doi.org/10.3109/02713689508999935>

## Challenges Between and Within Rural Minority Demographics

Benjamin Blue

University of California, Davis, Davis, CA, USA

**KEYWORDS:** Rural Communities, Health Disparities, Access to Care, Minorities, Social Determinants

---

**ABSTRACT:** Rural minorities in America are in poorer health compared to other demographics, as illustrated by deficits in several health metrics. Such deficits are great in magnitude and temporally persistent. Furthermore, the unrecognized diversity of rural areas means that these deficits affect a larger proportion of rural areas than conventional stereotype dictates. This review analyzes existing research to characterize the health of rural minorities, focusing on specific challenges and elucidating recurring themes that arise within and between minorities. Specific demographics were chosen for in-depth analysis based on prominence in nonmetropolitan areas, utility as case studies, and availability and quality of research. Population-specific analyses delineate themes that apply across demographics as well as challenges specific to each group, revealing the complexity of rural minority health. Analysis elucidates some key themes surrounding rural minority health. The invisibility of these demographics underlies many surface-level issues such as ineffective interventions and the underfunding of health organizations. Social context also plays a great yet underappreciated role in health, lending complexity to this issue through unique demographic-specific challenges. Above all, knowledge gaps are one of the most impactful and long-staying factors affecting rural minorities, impeding solutions and promoting disparity through ignorance on the part of both policymakers and providers.

---

### INTRODUCTION

The poor health of rural communities is well-established in literature, vividly illustrated by consistently reduced scores in health metrics. One example is urban-rural discrepancies in life expectancy. From 2006 to 2009, residents of nonmetropolitan counties were expected to live 76.8 years compared to 78.8 years in metropolitan counties [1]. Compounding this disparity is its stagnation; urban lifespans

have steadily increased over time, while rural lives have remained at their current length for decades [2]. Only half a year separated rural and urban lifespans from 1971 to 1974, but this gap quadrupled to 2.0 years from 2005 to 2009 [1]. Rural populations also have a higher prevalence of obesity than metropolitan populations [3,4]. With close ties to chronic, severe illnesses such as type 2 diabetes, high obesity rates imply poor health [5,6].

Rural mental health is challenged as well. In rural areas, all psychiatric disorders except for non-PTSD anxiety disorders are more prevalent [7]. Furthermore, from 1999 to 2016 in rural areas, drug overdose deaths increased by 749.4% and the suicide rate for those aged 25 to 64 years increased by 38.3% [8]. These statistics led the NIH to establish rural residents as a health-disparity population [9].

The healthcare struggles of rural areas are both distinct and difficult to overcome, rooted in the unique communities, cultures, and environments of a rural context. For instance, healthcare workers cite different obstacles in the workplace depending on their locality. Compared to urban providers, rural providers report struggling more with provider scarcities, finding continuing medical education, and staying compliant with the Health Insurance and Portability Accountability Act, a law that dictates acceptable usage and privacy protocols for medical records [10]. Rural culture is distinct as well, emphasizing family, traditionalism, trust, and independence. Cultural competence is paramount in rural health to gain patient trust, correctly and appropriately convey information, and foster accessibility [11]. However, proportionally few providers are recruited from rural backgrounds, promoting a cultural disconnect [12], facilitating the high patient-to-provider ratios that characterize rural healthcare [10]. Providers that fail to understand these values encounter community distrust and struggle to meet patient needs [13].

A defining struggle in rural health is long geographic distances. Compared to those in large metropolitan counties, five times as many rural households have no car and live further than a mile from a grocery store [1]. These vast distances spawn communication issues that hamper patient-provider contact, block cohesion between services, and damage efficiency,

all of which limit healthcare access, making accessibility a prominent issue in rural health [14,15]. Rural providers, already few in number, are spread thin over vast distances; lower service demand due to diffuse populations particularly affects specialists, who cluster in urbanized areas to support their practice [16]. High business expenses minimize profit margins for rural providers as well [17]. Large metro counties have 263 specialists per 100,000 people whereas rural counties see only 30 per 100,000, contributing to reliance on primary care [16]. With greater dependence on primary care, rural areas also suffer from the nationwide shortage of general practitioners, where 25-30 providers serve 100,000 people in both urban and rural areas [16].

Less well-understood, however, are the obstacles faced by minorities within rural communities. Despite a common stereotype of ethnic homogeneity, rural areas are home to significant proportions of vulnerable demographics and host a unique demographic makeup. The distinctions between rural and urban healthcare are mirrored by the distinctions between rural minorities and the majority. Furthermore, this diversity is growing due to the growth of low-wage industries. The number of minorities in rural areas increased by 20% between 2000 and 2010, while the number of rural Whites remained largely the same [18]. Just as rural communities have unique needs, cultures, and contexts compared to metropolitan areas, so do individual minority groups. Issues that independently plague rural and minority populations overlap in the doubly-underserved rural minorities, damaging patient outcomes [19]. Understanding these growing demographics is critical to serving their unique healthcare needs.

Medicine that understands and addresses the impact of rural minority standing

is better able to reach these vulnerable populations. This review will first identify several prominent demographics of rural areas: the disabled, Native Americans, veterans, and elderly. These are explored as illustrative case studies due to their high rural prevalence and availability of research. Their distinguishing factors are highlighted and, as these separations are identified, the overarching themes that unite these demographics emerge. Common ideas, observations, and challenges throughout rural minority health research are then explored. Keynote research motifs include ongoing research gaps on both rural health and rural minorities, leading to unaddressed issues and uninformed, ineffective policy. This contributes to another recurring theme, the invisibility of rural minorities in both public policy and the research world; the enduring struggles of these demographics is testament to this. Finally, the powerful negative effects of overlapping minority statuses continuously appears throughout research. Concluding this review is an analysis of these research themes and current deficiencies in our knowledge of these complex demographics.

### **OVERVIEW OF RURAL MINORITY HEALTH**

The rural-urban dichotomy in geography, culture, and historical context has been well-described with demonstrated, quantifiable impacts on health. However, distinctions between rural demographics are less recognized. For example, ethnic disparities in rural communities remain largely overlooked and undiscussed in literature [20]. Non-racial and non-ethnic demographic factors, such as the traits that define veteran, elderly, and disabled groups, are equally forgotten, rendering these groups invisible. Small population sizes and lack of representation in healthcare further obscure rural minorities, and underrepresentation

of the rural perspective among medical providers and researchers encourage stereotypes of homogeneity. This melange of factors occludes the recognition of specific needs and contextual factors both in literature and in medical practice. This theme of unacknowledged distinctiveness underpins rural minority health research.

Such lack of recognition conceals the desperate status of rural minority health. A strong recurring theme in literature is how intersecting geographic and minority disadvantages damage patient outcomes. As medically-under-served groups make up great amounts of rural populations, substantial amounts of people suffer from these intersections. For example, spatial isolation and low healthcare accessibility disproportionately impact rural minorities compared to their urban counterparts or the rural majority [20,21].

Compounding these common challenges are the unique struggles of individual groups, such as the powerful yet cryptic effects of social and cultural contexts. Apostolopoulos et al. see syndemics as the defining factor of rural minority health, describing the pathogenic interaction of biological and social factors [22]. The effects of syndemics appear frequently in literature; even cursory reviews of rural minority research underscore their obvious strength. For example, among Black Americans, several struggles – structural racism, inequitable social, education, labor, and health policies, and economic – are linked to trauma, stress, and syndemics [22]. Rurally, research regarding the magnitude of hardship for minorities takes disproportionately little notice of these contextual effects. This invisibility amalgamates with other factors, such as high and increasing rates of rurally-concentrated poverty and high employment in dangerous industries like manufacturing, to injure rural minority health [23]. Furthermore, for non-white rural residents,

the white-dominated rural setting means that specific cultural needs and experiences find little understanding. Coethnic networks and communities are sparse due to diffuse populations, and culturally-intelligent resources are few and far between. A prime example is in the rural Native American community, which struggles with fragmented healthcare services, low funding, and lack of community support [16]. Unsurprisingly, its members experience remarkably poor health even compared to other rural underserved [2].

All of these factors combine to produce visible, quantifiable damage to health. Rural minorities have lower life expectancies, see higher mortality rates, rate their healthcare as lower quality, receive fewer preventative services, and suffer higher rates of chronic disease compared to their urban counterparts [6,24]. Table 1 summarizes health statistics in rural ethnic and racial demographics [20].

The complex aggregation of social, cultural, and geographic factors generates the current healthcare plight of rural minorities. “Health outcomes are inexorably linked to ZIP code, are notably worse for the millions who live in rural and underserved communities and are further compounded by health disparities common among racial and ethnic groups.” [21] Interventions to address social determinants of health are equally as important as secondary prevention and treatment efforts yet remain underutilized in policy due to poor recognition of these determinants. [25] Research is limited for all rural minorities, but available literature reveals the distinctive qualities of each group as well as overarching patterns that unite them. Understanding the interplay of unique challenges and broader factors will allow for more powerful interventions in these underserved communities.

## **NATIVE AMERICANS**

Native Americans exemplify the struggles of rural minorities, experiencing some of the poorest health outcomes in America despite their intimate ties to rural land: in 2010, 29% of Native Americans lived in rural areas compared to 15% of the U.S. population [26]. Furthermore, where other rural ethnicities have experienced gradual but measurable gains in healthcare access and quality, lingering historical biases and social factors continue to negatively affect Native American health [27].

Notably, rural Native Americans suffer one of the highest risks of maternal and infant mortality in the U.S [21,28], high rates of mental illness, stress [27], substance abuse [29], and suicide [24], and damaged mental well-being as well [4]. Native Americans also suffer higher mortality rates of preventable disease. Per 100,000 people, pneumonia and influenza kill 26.6 Native Americans per year versus 15.1 for all races, and heart disease kills 194.7 Native Americans as opposed to 179.1 for all races [29]. Unsurprisingly, the rural-urban disparity in life expectancy is exaggerated for Native Americans, where a gap of 11 years stands between metropolitan and nonmetropolitan Indigenous lifespans [1]. Economic obstacles hinder Native healthcare, as well: 33% of rural Native Americans report recent problems accessing healthcare, and 28% recently experienced major problems paying medical bills. High costs do not indicate high quality, either, as 28% of rural Native Americans reported recent problems with quality of healthcare [30].

Perhaps one of the greatest issues faced by this group is healthcare fragmentation. “Services and support for health and social programmes are typically fragmented in Indigenous populations... Fragmentation results in the isolation of symptomatic issues—addiction, suicide, fetal alcohol syndrome, poor

housing, and unemployment—followed by the design of stand-alone programmes to try to manage each issue separately.” [31] Fragmentation is bred by chronic underfunding, small populations, and long geographic distances [32], and as a population known for its small size and economic hardship, Indigenous health is the paragon of such fractionation. Fragmented healthcare services interrupt cohesion between services, leading to poor outcomes, inaccessibility, and inefficient resource use [33]. The Indian Health Service (IHS), which only provides non-comprehensive health insurance and experiences chronic underfunding, suffers extensively from fractionation [29]. As many rural American Indians/Alaska Natives receive healthcare through the IHS (approximately 1.5 million of 4.1 million Native Americans [34]), a great number of rural Indigenous encounter fractionated, underfunded healthcare: 46% of American Indian/Alaskan Natives receiving care through the IHS experienced funding shortfalls and subsequent reductions in healthcare [29].

More factors than just the IHS affect rural Native American healthcare, such as stark socioeconomic status. Compared to the total U.S. population, Native Americans working full-time earned less and were more likely to live in poverty compared to non-Hispanic whites [26]. There are also substantial differences between the experiences of rural Native Americans and whites not only in healthcare discrimination, but also in police treatment, racial violence, and housing [30]. Health outcomes are not produced in a vacuum; social factors influence healthcare at all levels, from policymaking to patient-provider interactions, to generate palpable impacts [21,25]. Rural Black and Hispanic Americans suffer the same pattern, where structural racism influences health to damage outcomes [35]. From the poorer healthcare out-

comes across all rural ethnic minorities, we see how minority standing in majority-white rural areas affects patient outcomes. Interventions in rural Indigenous healthcare struggle to address these. Prominently, even the introduction of the IHS, specifically created to address Native American healthcare, made little change in Native American healthcare over time [29]. Other interventions outside of the IHS are primarily targeted at individuals and communities [63]. While helpful, the greater systemic pressures on rural Native Americans remain, thus no substantial, national change in Indigenous health has been accomplished.

Rural Native Americans' twice-erased status in locality and ethnicity contributes to their current healthcare struggles. Thus, the healthcare effects of such a unique context are forgotten. As indigeneity is an inherently social and cultural concept, poor Indigenous health is rooted in cultural factors such as loss of community, lack of land connection, and feelings of spiritual, emotional, and mental disconnectedness [31]. This disconnection is a great mental burden: the rural Indigenous experience the highest suicide rate out of all rural demographics, double that of rural whites and twelve times that of rural African- and Asian-Americans [24]. The fractionated state of rural Native healthcare mirrors this disconnection. Overall, the rural Indigenous have not received the benefit of medical advancements, leading to stagnated improvement in health [21].

The pervasive stereotype of rural communities as homogenous oversimplifies them, rendering the diverse experiences of ethnic and racial constituents invisible. Such invisibility affects healthcare at all levels, from public health initiatives and funding to patient-provider interactions. This invisibility is woven throughout rural health research, not just among the Native American population. Other

rural health researchers have commented on the difficulty of finding high-quality samples, trends, and pre-existing research. The issues dogging the unseen rural Indigenous -- economic, social, and cultural, not just in health-care -- have halted improvement in their health as others advance.

## DISABILITY

Rural populations have markedly high proportions of disability. Compared to metropolitan adults, rural adults are 9% more likely to report having any disability, 24% more likely to report having more than one disability [36], and are more likely to have hearing or vision loss [4]. Low workforce participation and economic constraints facilitate the high rates of poverty experienced by disabled households [37]. Despite the substantial presence of disability in rural areas, the rural disabled are a rare topic in healthcare research [21].

Accessibility frames the daily lives of disabled people. In a healthcare context, disability management and quality of life are contingent on accessible, high-quality care, as the disabled community requires more health services than abled persons [38]. Thus, rural scarcity of both specialty and primary care, a necessity in the lives of many disabled people, disproportionately impacts this population.

Geographic inaccessibility defines rural areas, where vast distances between services make car travel essential. As driving requires well-functioning sight, hearing, cognitive ability, and mobility, many disabled people cannot drive long distances and struggle to access care. Provider scarcities build only more obstacles, as many disabled people struggle to locate specialist care for disability-specific services [38]. The vacuum of nonurban specialty care impacts the health of the rural disabled, who need these services most. Rural residents

rely heavily on primary care providers as well, even to manage conditions largely overseen by specialists [12]. Regardless of the fact that primary care cannot provide the same services as a specialist, the shortage of rural primary care inordinately affects the rural disabled, for whom consistent access to healthcare is critical. Low socioeconomic status magnifies the effect of provider scarcity. Many disabled adults (42.0%) depend on Social Security Disability Insurance or Supplemental Security Income as well, including services such as Medicare [38]. As these public programs pay around half of what private insurance pays practitioners [39], providers have less incentive to care for rural disabled, further limiting care options. Financial barriers also impede access to care: working-age adults with disabilities are much more likely than those without to report inability to pay medical bills, problems paying medical bills, and not accessing care due to cost [38].

A recurring theme throughout rural minority healthcare is the numerous overlaps between marginalized populations. Few demographics exemplify this better than rural disability. Non-White populations [37] and veterans experience higher rates of disability and veterans have high rates of disability as well [40]. The elderly often encounter disability as they age, and healthcare obstacles in these separate populations frequently overlap, as in transportation struggles, high incidence of poverty, and dependence on public programs. Overall, frequent overlaps with underserved groups results in rural disability generating more destitute outcomes.

Disability and rurality have a close relationship that directly affects healthcare and quality of life. Several layers of inaccessibility hinder rural health, from financial to geographic, and the overlap of disability status with other individual factors lends further com-

plexity to rural disabled healthcare. However, research into the rural disabled is noticeably lacking despite a critically underrepresented status. For example, the interplay of disability with other minority standings is known in only very broad, general trends. Financial impacts remain understudied as well. Further research to characterize rural disability is needed to fully understand its effects and its intersections with other groups.

### VETERANS

Rurality and veteranship are closely tied, with approximately 4.7 million veterans currently living in rural and highly rural areas [41]. Unsurprisingly, the issues that plague rural healthcare merge with veteran-specific traits to damage rural veteran health, resulting in low health-related quality-of-life scores for rural veterans compared to their nonrural counterparts [42]. Institutional underfunding and high intrinsic rates of disability are some of the prominent factors affecting the health of rural veterans.

Similar to the struggles of the IHS, the Veterans Health Administration (VHA) has seen regionalization and cutting of rural services due to small service populations and limited finances [7,43]. Dilute service populations require the establishment of large referral regions and, as a result, VHA tertiary care referral centers tend to be located in urban areas even if many clients are rurally-located [43]. This creates referral systems that are geographically separated from patients. For veterans, this disconnection exacerbates foundational issues in rural healthcare, generating long travel times and augmenting the lack of specialty care. As illustrated in the rural Indigenous, fractionation and defunding of healthcare is common among the rural underserved and damages healthcare outcomes.

As found above, disability frequently intersects with other minority statuses to im-

pact health, exemplified by the high rates of disability among veterans. 29% of veterans are classified by the US government as having a service-connected disability [40] compared to the general disability rate of 4.3% [44]. Service-connected disabilities include physical maladies as well as mental illness, with mental illness being a defining veteran's issue. Of veterans utilizing Veterans Affairs health services, 21.8% are diagnosed with post-traumatic stress disorder and 17.4% are diagnosed with depression, proportions much higher than in civilians [44]. High disability rates affect more than just health, contributing to, for example, high rates of household poverty. 13.19% of disabled veterans are in poverty compared to 5.51% of non-disabled veterans, damaging quality of life and introducing financial barriers to healthcare [37].

Mental illness is a prominent veteran struggle due to traumatic experiences and military culture discouraging the use of behavioral healthcare. Veteran suicide rates increased by 15% for men and 35% for women from 2001 to 2010 [45]. Rural veterans struggle even more: physical and mental health scores are far lower for nonurban veterans compared to urban veterans, showing the larger burden of mental illness for rural veterans [7]. The lack of care sensitive to veterans' particular needs and context, such as for mental health, fuels these struggles. If rural healthcare is sparse in general, there are less informed on the specific circumstances of veterans. Military culture, with values of strength and independence, impacts how veterans approach healthcare; effective healthcare for veterans is sensitive to these cultural considerations and the distinct obstacles veterans face [40].

Though veteranship holds unique contextual considerations for healthcare, recurring challenges across minorities impact

this population. Demographic-specific healthcare organizations struggle to support diffuse rural populations, demonstrated in rural Native Americans with the IHS and in veterans with the VHA. Similarly, the lack of veteran-specific care echoes the cultural gaps that impact the rural Indigenous. Predominately white providers struggle with the specific issues surrounding the Indigenous; metropolitan providers struggle to fully appreciate rural culture; the effects of military culture often go unrecognized by civilian providers. Military values emphasize “the physical and mental well-being of soldiers, as well as their independence and self-reliance...” discouraging veterans from seeking healthcare [40]. Nonmilitary providers unaware of these considerations thus struggle to reach veterans. Coupled with rural obstacles to care, there is little incentive for veterans to pursue healthcare, facilitating health disparity.

Rural veterans, by virtue of both their locality and life experiences, represent a deeply underserved population. Thinly-spread health services, lack of funding, high disability and poverty rates, poorly-defined referral areas, and care uninformed on military culture combine to generate uniquely poor health circumstances for rural veterans. This mixture of struggles incorporates wider issues in rural health and unique contextual obstacles. The poorer health of veterans, reflected in high suicide rates and reduced quality of life, is rooted in many different factors, difficult to untangle from each other but powerful in their combined effect.

## **ELDERS**

Quiet, idyllic, and slow-paced, rural locales make popular retirement destinations. 25% of the rural population is age 65 or older, though the elderly constitute 12% of the U.S. population [48]. More Americans are reaching

retirement age and increasing this proportion, foreshadowing growth of current issues with provider shortages [49]. The effects of this shortage are compounded by the intensive healthcare needs of elderly patients. The medical intricacy of aging merges with issues in service access and provider shortages to establish the elderly as a medically vulnerable population. Rural geography and overlaps with other minority statuses only exacerbate these issues.

Elderly patients benefit from specialty healthcare that is trained in complex and aged patient care [48,50]. However, a drastic shortage in geriatricians is developing, especially in rural areas [51]: for example, 56% of geriatric fellowship spots remained unfulfilled in the 2015 Main Residency Match of the National Resident Matching Program [52]. Rural elders thus suffer not just from broad deficiencies in rural specialty care, such as cardiology for age-related heart disease, but for a demographic-specific specialty trained in their specific needs. Mental health is also severely underemphasized in the elderly. Social and spatial isolation plague the rural elderly [49], yet psychological services to cope with such loneliness are scarce [54]. Suicide risk increases significantly after age 65, and 18% of completed suicides in 2019 were by older Americans [55]. Aside from specialty care, rural elders encounter pronounced challenges in primary care access, such as physical frailty and long geographic distances [5]. Compounding this hardship is insufficient public transportation in rural areas [17].

Care that would circumvent these physical barriers, such as home-based support services, is lacking. Limited provider availability, inadequate transportation, poor telecommunications access (i.e. poor Internet and technological literacy in elders), and low caregiver

recruitment and retention plague these services [14,17]. The same issues that haunt all of rural healthcare impact home health, damaging the health of rural elders who need these services most.

Socioeconomic factors also impact the elderly. High poverty rates in rural elders introduce financial barriers to care. Compared to urban areas, rural elders have lower incomes and are more likely to be below the poverty line. Although they are more likely to own their homes, those homes are more likely to be substandard [55]. Greater reliance on Medicaid and Social Security may also limit provider options [55]. Long distances to work or support services only worsen financial issues. Overall, vast distances may impose insurmountable barriers to healthcare, employment, prescriptions, or social services for rural elders [56].

As American lifespans stretch longer and more retirees seem to seek calm, idyllic towns and views, healthcare in the rural elderly will only become more important. These elders, however, will find only sparse healthcare services, whether in primary care, specialty care, or home health. Frequent intersections with other marginalized statuses mar the health outcomes of rural elderly as well. For example, 56% of the 2.8 million rural veterans enrolled in the VHA are over the age of 65 [57]. Furthermore, the proportion of ethnic and racial minority groups among the American elderly is expected to rise sharply in coming years: the percentage of elderly non-Hispanic whites is expected to drop from 78% in 2014 to 55% in 2060 [49]. Throughout rural health, overlapping circumstances facilitate poor outcomes, and rural elders follow the same pattern [56]. Little research has been completed about these overlaps, however, leaving a number of unanswered questions.

## ONGOING KNOWLEDGE GAPS

The experiences of rural minorities are important to consider in rural health due to the surprising heterogeneity of rural areas. Factors that independently affect minority and rural populations overlap to damage healthcare quality and access. The intricacy and significant numbers of rural minorities make them critical aspects of rural areas, yet are often overlooked. Current research into rural minority health highlights unique challenges of each demographic and implies universal challenges that unite them. Some overarching patterns include the magnification of extant rural health issues for minorities, socioeconomic obstacles, and underfunded programs for specific demographics. Of these patterns, persistent invisibility in their own communities and healthcare itself is the most consistent and impactful. Such invisibility camouflages the impact of rural minority standing on health, causing it to be overlooked and understudied.

Large, ongoing gaps in our knowledge unify rural minority research, with rural Native Americans being a prime example of these gaps. The Indigenous are a group already poorly-understood, with the rural Indigenous only doubly so. Small samples and inconsistencies in research methods, such as how tribes and ethnic groups are merged, make the Indigenous difficult to research and contribute to the lack of literature surrounding them [33]. Baldwin et al. report that, even for a basic measure such as mortality rate, “few studies have reported [American Indian/Alaska Native] mortality nationally and stratified by residence location.” [58] Other minorities suffer the same invisibility. For the disabled community, little is known about disability prevalence and types of disability by urbanization level [36]. For rural elders, demographic-specific issues such as elder mistreatment are known

to be understudied, and such issues affect rural elders even more due to geographic obstacles in knowledge and intervention efforts [49]. As for veterans, there is little information on current suicide rates, let alone historical trends, who make up great proportions of rural areas [45]. Multiple-minority demographics are doubly affected by these gaps and remain chronically understudied.

Knowledge gaps are particularly wide in rural minority mental health. This is deeply concerning for a number of reasons. As both rural and minority populations experience separate shortages in mental health services, a poor understanding of these overlaps is deeply detrimental [15]. Jensen et al. argue that rural areas require stronger interventions to overcome mental health deficits, as the combination of a distinct culture and strong, established barriers to behavioral health care damage accessibility and quality [59]. They echo King et al. who, in reference to rural Native Americans, argue that further research into social determinants of health will form better interventions [31]. Across demographics and healthcare specialties, research agrees that a broad lack of research makes effective solutions difficult to form.

More broadly, research into rural healthcare itself is lacking. Many studies are limited to phone surveys or analyses of aggregated hospital data, a shaky choice of data collection considering the rapid closures of rural hospitals [60] and rural dependence on small clinics and private practices. General consensus holds that rural health is poorer than that in urban areas, but large-scale studies in specific health indicators are scarce. For example, even as the US has defined increasing life expectancy as a key goal [61], few studies analyze rural-urban life expectancy disparities and factors affecting lifespan. Literature examining the plight of rural specialty care is also diffuse. Cyr et al. find an

“...insufficient understanding of differences in facilitators and barriers between U.S. urban versus rural specialty care. While conceptual frameworks exist to guide these efforts... none specifically focus on US urban versus rural specialty care.” [62] A lack of research leaves the details of rural health unexplored.

The depth and persistence of these knowledge gaps may render interventions in rural minority health ineffective. For example, Apostolopoulos et al. identify several common yet low-leverage interventions (e.g., self-management education) often employed in rural communities that generate underwhelming results [22]. Our thready understanding of rural areas, frequently informed by stereotypes and assumptions, obscures more effective solutions. Thus, the health of the rural underserved stagnates and declines.

Previous literature unanimously agrees that rural communities and their substituent minority groups are distinct from both urban populations and each other. Each demographic has particular needs, values, and problems influenced by unique contextual and cultural factors; however, research has not fully studied the effect of intersecting minority statuses. Such research gaps preclude a complete understanding of the unique context of minorities, impeding the implementation of solutions to uplift individual demographics and rural communities as a whole. A richer understanding of the rural minority experience -- their unique, defining factors, their prevalence, and their historical context -- will illuminate effective solutions and additional factors to bridge the gap between them and their urban counterparts. Targeted research into rural minorities is a direct pathway to this deeper understanding. As shown in literature, there are several unanswered questions about the specific challenges rural minorities face, occluding effective solu-

tions. There is a wealth of information still to be gained about these highly complex, incredibly interesting groups. Greater research efforts will increase public awareness and direct effective interventions, creating decisive changes to overcome these ongoing challenges.

**FIGURES**

| Metric   | Black % | Hispanic % | Native American % | White % |
|--|---------|------------|-------------------|---------|
| Health status: fair or poor                              | 28.8*   | 28.4*      | 28.9*             | 18.5    |
| Frequent physical distress                               | 15.9*   | 13.9       | 19.6*             | 13.3    |
| Frequent mental distress                                 | 13.9*   | 11.2       | 17.1*             | 12.5    |
| Could not see a doctor in the post 12 months due to cost | 24.5*   | 23.1*      | 19.1*             | 15.0    |
| Have at least one personal doctor/provider               | 73.2*   | 61.5*      | 63.7*             | 78.6    |

Table 1. Health Metrics of Rural Demographics, Reporting Period 2012 - 2017  
 Selected data concerning healthcare access and health of rural demographics. \* indicates significant difference from non-Hispanic white. Adapted from James et al.

**AUTHOR INFORMATION**

**Corresponding Author**

Benjamin Blue (University of California, Davis)  
 amblue@ucdavis.edu

**Author Contributions**

Benjamin Blue, as primary author, performed all research and preparation of this manuscript.

**Funding Sources**

No external funding was used in the preparation of this manuscript.

**Competing Interests**

The author declares no competing financial and non-financial interests.

**REFERENCES**

[1]. G. Singh, M. Siahpush, Widening rural–urban disparities in life expectancy, U.S., 1969–2009. *Am. J. of Preventive Medicine* 46.2, E19-E29 (2014).

[2]. J. Saint Onge, S. Smith., Demographics in rural populations. *Surg. Clin. North Am.* 100.5, 823-833 (2020).

[3]. K. Matthews et al., Health-related behaviors by urban-rural county classification — United States, 2013. *MMWR Surveill. Summ.* 66.5, 1-8 (2017).

[4]. J. Schiller, J. Lucas, J. Peregoy, Summary health statistics for U.S. adults: National Health Interview Survey, 2011. *Vital Health Stat.* 10.256, 1-218 (2011).

[5]. J. Dixon, “The effect of obesity on health outcomes.” *Mol. Cell. Endocrinol.* 316.2, 104-108 (2010).

[6]. America’s Health Insurance Plans, “Serving Rural America: How Health Insurance Providers Break Down Barriers to Ensure Access to Care.” (AHIP Reports, 2019).

[7]. A. Wallace, W. Weeks, S. Wang, A. Lee, L. Kazis, Rural and urban disparities in health-related quality of life among veterans with psychiatric disorders. *Psych. Serv.* 57.6, 851-856 (2006).

[8]. S. Woolf, H. Schoomaker, Life expectancy and mortality rates in the United States, 1959-2017.” *JAMA* 322.20, 1996-2016 (2019).

- [9]. National Institute of Health, "NIH health disparities strategic plan and budget: Fiscal years 2009-2013." (National Institute of Health, 2009). Available from [https://www.nimhd.nih.gov/docs/2009-2013nih\\_health\\_disparities\\_strategic\\_plan\\_and\\_budget.pdf](https://www.nimhd.nih.gov/docs/2009-2013nih_health_disparities_strategic_plan_and_budget.pdf).
- [10]. L. Hart, E. Larson, D. Lishner. Rural definitions for health policy and research. *Am. J. Public Health* 95.7, 1149-1155 (2005).
- [11]. T. Thomas, R. DiClemente, S. Snell, Overcoming the triad of rural health disparities: How local culture, lack of economic opportunity, and geographic location instigate health disparities. *Health Ed. J.* 73.3, 285-294 (2013).
- [12]. R. Rosenblatt, F. Chen, D. Lishner, M. Doescher, "The future of family medicine and implications for rural primary care physician supply." Final report #125. (WWAMI Rural Health Research Center, 2010).
- [13]. S. Letvak, The importance of social support for rural mental health. *Issues Ment. Health Nurs.* 23.3, 249-261 (2002).
- [14]. K. Gonzalez, M. Shaughnessy, E. Kabigting, D. West, J. Robinson, Q. Chen, P. Fahs, A systematic review of the health of vulnerable populations within U.S. rural societies. *Online J. Rural Nurs. Health Care* 18.1, 112-147 (2018).
- [15]. D. Morales, C. Barksdale, A. Beckel-Mitchener, A call to action to address rural mental health disparities. *J. of Clin. Transl. Sci.* 4.5, 1-20 (2020).
- [16]. M. Meit et al., "The 2014 update of the rural-urban chartbook." (Rural Health Reform Policy Research Center, 2014).
- [17]. D. Siconolfi et al., Rural-urban disparities in access to home- and community-based services and supports: stakeholder perspectives from 14 states. *J. Am. Med. Dir. Assoc.* 20.4, 503-508 (2019).
- [18]. D. Lichter, Immigration and the new racial diversity in rural America. *Rural Sociol.* 77.1, 3-35 (2012).
- [19]. K. Kozhimannil, C. Henning-Smith, Racism and health in rural America. *J. Health Care Poor Underserved* 29.1, 35-43 (2018).
- [20]. C. James, R. Moonesinghe, S. Wilson-Frederick, J. Hall, A. Penman-Aguilar, K. Bouye, Racial/ethnic health disparities among rural adults — United States, 2012–2015. *Surveillance Summaries* 66.23, 1-8 (2017).
- [21]. Rural and Underserved Communities Health Task Force, "Left out: barriers to health equity for rural and underserved communities." (U.S. House of Representatives Committee on Ways and Means, 2020).
- [22]. Y. Apostolopoulos, M. Lemke, N. Hosseinichimeh, I. Harvey, K. Lich, J. Brown, Embracing causal complexity in health disparities: metabolic syndemics and structural prevention in rural minority communities. *Prev. Sci.* 19.8, 1019–1029 (2018).
- [23]. T. Farrigan, T. Parker, The concentration of poverty is a growing rural problem. *Amber Waves* 4 (2012).
- [24]. Web-Based Injury Statistics Query and Reporting System, Fatal injury reports. Centers for Disease Control and Prevention. Available at <https://www.cdc.gov/injury/wisqars/fatal.html>. Accessed 11/14/2021.

- [25]. R. Thornton, C. Glover, C. Cené, D. Glik, J. Henderson, D. Williams, Evaluating strategies for reducing health disparities by addressing the social determinants of health. *Health Aff.* 35.8, 1416–1423 (2015).
- [26]. S. Ogunwole, “We the people: American Indians and Alaska Natives in the United States.” (U.S. Census, 2006).
- [27]. J. Waldram, A. Herring, T. Young, *Aboriginal Health in Canada* (University of Toronto Press, 2006).
- [28]. D. Ely, A. Driscoll, Infant mortality in the United States, 2018: Data from the period linked birth/infant death file. *National Vital Statistics Reports* 69.7, 1-17 (2020).
- [29]. D. Warne, L. Frizzell, American Indian health policy: Historical trends and contemporary issues. *Am. J. Public Health* 104.3, S263–S267 (2014).
- [30]. M. Findling, R. Blendon, J. Benson, C. Miller, The unseen picture: issues with health care, discrimination, police and safety, and housing experienced by Native American populations in rural America. *J. Rural Health* 36, (2020).
- [31]. M. King, A. Smith, M. Gracey, Indigenous health part 2: The underlying causes of the health gap. *Lancet* 374.9683, 76-85 (2009).
- [32]. Council on Graduate Medical Education, “Physician distribution and health care challenges in rural and inner-city areas.” (Department of Health and Human Services, 1998).
- [33]. L. Trout, C. Kramer, L. Fischer, Social medicine in practice: realizing the American Indian and Alaska Native right to health. *Health Hum.* 20.2, 19-30 (2018).
- [34]. M. Cahn, S. Harvey, M. Town, American Indian and Alaska Native men's use of sexual health services, 2006-2010. *Perspectives on Sexual and Reproductive Health* 49.3, 181-189 (2017).
- [35]. D. Nguyen, K. Ho, J. Williams, Social determinants and health service use among racial and ethnic minorities: findings from a community sample. *Soc. Work Health Care* 50.5, 390-405 (2011).
- [36]. G. Zhao, C. Okoro, J. Hsia, W. Garvin, M. Town, Prevalence of disability and disability types by urban–rural county classification—U.S., 2016. *Am. J. Prev. Med.* 57.6, 749-756 (2019).
- [37]. A. London, C. Heflin, J. Wilmoth, Work-related disability, veteran status, and poverty: implications for family well-being. *J. Poverty* 15.3, 330-349 (2011).
- [38]. J. Kennedy, E. Wood, L. Frieden, Disparities in insurance coverage, health services use, and access following implementation of the affordable care act: a comparison of disabled and nondisabled working-age adults.” *Inq.* 54, 1-10 (2017).
- [39]. E. Lopez, T. Neuman, L. Levitt, G. Jacobson, How much more than medicare do private insurers pay? A review of the literature. (KFF, 2012). Available at <https://www.kff.org/medicare/issue-brief/how-much-more-than-medicare-do-private-insurers-pay-a-review-of-the-literature/>.
- [40]. C. Griffin Jr., M. Stein, Self-perception of

- disability and prospects for employment among U.S. veterans. *Work* 50.1, 49-58 (2014).
- [41]. Veterans Affairs Office of Rural Health, Rural veteran healthcare challenges. (Veterans Affairs, 2012). Available at <https://www.rural-health.va.gov/aboutus/ruralvets.asp>
- [42]. A. Wallace, R. Lee, T. MacKenzie, A. West, S. Wright, B. Booth, K. Hawthorne, W. Weeks, A longitudinal analysis of rural and urban veterans' health-related quality of life. *J. Rural Health* 26.2, 153-163 (2010).
- [43]. R. Tessler, R. Rosenheck, G. Gamache, Declining access to alcohol and drug abuse services among veterans in the general population. *Military Medicine* 170, 234-238 (2005). U.S. Census Bureau, 2020 Census ACS 1-year data. (U.S. Census Bureau, 2020).
- [44]. K. Seal, D. Bertenthal, C. Miner, S. Sen, C. Marmar, Bringing the war back home: Mental health disorders among 103,788 US veterans returning from Iraq and Afghanistan seen at Department of Veterans Affairs facilities. *Arch. Intern. Med.* 167.5, 476-482 (2007).
- [45]. J. McCarten, C. Hoffmire, R. Bossarte, Changes in overall and firearm veteran suicide rates by gender, 2001–2010. *Am. J. Prev. Med.* 48.3, 360–364 (2015).
- [46]. A. Skoufalos, J. Clarke, E. Ellis, V. Shepard, E. Rula, Rural aging in America: proceedings of the 2017 connectivity summit. *Popul. Health Manag.* 20.S2, S1-S10 (2018).
- [47]. Institute of Medicine. *Retooling for an aging America: building the health care workforce* (National Academies Press, 2008).
- [48]. C. Dyer, C. Murdock, J. Hiner, J. Halphen, J. Burnett, Elder mistreatment intervention: strategies for connecting with diverse and rural populations. *Generations* 44.1, 91-97 (2020).
- [49]. A. Hintenach, O. Raphael, W. Hung, Training programs on geriatrics in rural areas: a review. *Curr. Geriatr. Rep.* 8.2, 117-122 (2019).
- [50]. A. Golden, M. Silverman, M. Mintzer, Is geriatric medicine terminally ill? *Ann. Intern. Med.* 156, 654–656 (2012).
- [51]. National Resident Matching Program, “Results and data: Specialties Matching Service, 2015 appointment year.” (National Resident Matching Program, 2015). Available from <http://www.nrmp.org/wp-content/uploads/2015/02/Results-and-Data-SMS-2015.pdf>.
- [52]. M. Kaplan, N. Huguet, B. McFarland, J. Mandle, Factors associated with suicide by firearm among U.S. older adult men. *Psychol. Men Masc.* 13.1, 65-74 (2012).
- [53]. R. Hudson, Lack of social connectedness and its consequences. *Public Policy & Aging Report* 27.4, 121–123 (2017).
- [54]. A. Coburn, E. Bolda, Rural elders and long-term care. *West. J. Med.* 174.3, 209-213 (2001).
- [55]. B. Collins, T. Borders, K. Tebrink, K. Xu, Utilization of prescription medications and ancillary pharmacy services among rural elders in west Texas: distance barriers and implications for telepharmacy. *Journal of Health and Human Services Administration* 30.1, 75-97 (2007).
- [56]. Department of Veterans Affairs, “FY2020

Funding and FY2021 Advance Appropriations, Volume II Medical Programs and Information Technology Programs.” (Department of Veterans Affairs, 2019).

[57]. A. Scharlach, E. Fuller-Thomson, B. Kramer, “Curriculum model on aging and ethnicity.” (University of California Berkeley School of Social Welfare, 2002).

[58]. L. Baldwin, D. Grossman, S. Casey, W. Hollow, J. Sugarman, W. Freeman, L. Hart, Perinatal and infant health among rural and urban American Indians/Alaska Natives. *Am. J. Public Health* 92.9, 1491–1497 (2002).

[59]. E. Jensen, E. Wieling, T. Mendenhall, A phenomenological study of clinicians’ perspectives on barriers to rural mental health care. *J. Rural Ment. Health* 44.1, 51-61 (2020).

[60]. B. Kaufman, S. Thomas, R. Randolph, J. Perry, K. Thompson, G. Holmes, G. Pink, The rising rate of rural hospital closures. *The Journal of Rural Health* 32.1, 35-43 (2016).

[61]. Healthy People 2020. Healthy People 2020. (Office of Disease Prevention and Health Promotion, 2014). Available at <https://www.healthypeople.gov/2020>.

[62]. M. Cyr, A. Etchin, B. Guthrie et al., Access to specialty healthcare in urban versus rural US populations: a systematic literature review. *BMC Health Serv Res* 19, 974 (2019).

[63]. V. Blue Bird Jernigan, E. J. D’Amico, B. Duran, D. Buchwald, Multilevel and Community-Level Interventions with Native Americans: Challenges and Opportunities. *Prevention Science* 21, 65–73 (2018).