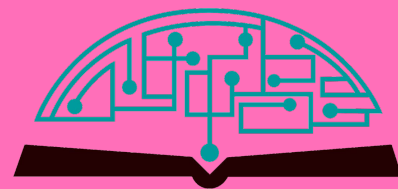


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An EEG-Based Neural Re-evaluation Index for Moral-Semantic Conflict

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ABSTRACT: Most studies of cognitive control rely on experiment-wide averages, failing to capture trial-by-trial neural adaptation essential for quantifying an individual's capacity for belief updating. Consequently, there is currently no standardized, survey-free EEG metric to operationalize re-evaluation readiness when individuals encounter conflicting information. This project aims to address this gap by developing the Neural Re-evaluation Index (NRI) to function as a physiological marker of cognitive re-evaluation capacity. The study utilized a publicly available BIDS-formatted EEG dataset with 31 participants that involved moral-semantic conflict. The frontal theta power and the theta-beta ratio (TBR) were extracted using a reproducible Python pipeline. The NRI was constructed by calculating the standardized modulation of these signals during incongruent "re-evaluation triggers" relative to baseline congruent trials and was validated using group-level statistical comparisons and Leave-One-Subject-Out (LOSO) cross-validated decoding. Results showed that incongruent stimuli displayed a higher frontal theta and TBR values at the group level, validating the NRI's sensitivity to conflict-related neural recruitment. The LOSO classifier achieved a modest AUC of 0.543, establishing a foundation for a discriminative signal for identifying belief-updating states while reflecting the inherent complexity of cross-subject neural decoding. These findings support theories of functional dissociation, where increased theta-beta dynamics indicate the active suppression of prior mental models. The utility of the NRI lies in its reproducible, EEG-only framework that bypasses the biases of self-report measures by capturing re-evaluation readiness at a neural level with broad applications for enhancing conceptual change in education and improving resistance to misinformation.

KEYWORDS: EEG, Bias, Neural Activity, Brain Waves, NRI.

■ Introduction

Electroencephalography, a non-invasive measurement of the brain's electric fields, is used to record the brain's electrical activity through electrodes, capturing brain waves.¹ Brain waves are oscillating electrical voltages in the brain, measuring just a few millionths of a volt.² These brain waves are classified by frequency into five bands: delta (0.5–4 Hz), theta (4–7 Hz), alpha (8–12 Hz), beta (16–31 Hz), and gamma (36–90 Hz).³ The five brainwave bands represented distinct ranges of neural oscillations, each associated with characteristic functional states of the brain, from deep sleep and relaxation to active cognition and heightened concentration.⁴

As a result of EEGs' millisecond-level temporal resolution and comparatively low cost to other neuroimaging modalities, it has been widely adopted in both clinical and research contexts worldwide.¹ Clinically, EEG is used to diagnose and monitor neurological conditions, while at the research level, it has become central to the study of cognition, perception, and adaptive behaviour.⁵ Globally, EEG constitutes a standard diagnostic and monitoring tool in neurology for conditions such as epilepsy, sleep disorders, and encephalopathies.⁶

Moreover, EEG has also been extensively applied to the study of error processing and decision-making due to its ability to capture rapid neural responses to cognitive conflict and feedback. The growth of open-access neuroimaging repositories supported these advances.⁷ In India, researchers at institutions like AIIMS Raipur and AIIMS Kalyani used EEG and ERPs

to objectively identify subclinical cognitive impairment in PCOS patients by measuring markers such as increased theta-alpha ratios and reduced P300 amplitudes.⁸ Another study employs high-density EEGs to demonstrate that faces act as more potent emotional distractors than words, activating neural circuits associated with saliency, conflict, and goal-directed behaviour.⁹

Research has consistently demonstrated that distinct EEG frequency bands were differentially involved in decision-making, evaluation, and self-referential cognition. For example, a study investigating intracranial activity found that cortical theta and beta oscillations in the lateral prefrontal cortex (LPFC) functionally dissociate during decision-making, with higher theta activity observed during impulsive choices and higher beta activity during non-impulsive decisions.⁸ A paper on social dynamics revealed that low-dominance individuals exhibit larger Feedback-Related Negativity (FRN) amplitudes, indicating a heightened concern for performance evaluation and sensitivity to negative prediction errors.¹⁰ The study defined low dominance using the Personality Research Form dominance subscale (PRF-d). Another investigation into memory recall demonstrated that recalling decisions linked to self-threatening negative feedback requires greater cognitive effort, manifesting as increased beta and gamma band activity.¹¹ Additionally, a study on motivation identified Frontal Alpha Asymmetry (FAA) as a key predictor of decision-making, where leftward

asymmetry serves as a proxy for withdrawal processes, while rightward asymmetry reflects an approach-oriented state.¹²

Technique-focused EEG research has emphasized the development of composite frequency-based indices to quantify cognitive states with greater precision. An example is the Theta-to-Alpha Ratio (TAR), which is a highly effective marker for measuring cognitive effort and workload during memory manipulation tasks.¹² In contrast, the Theta-Alpha-to-Beta Ratio (TA2BR) measured at temporal locations has emerged as a superior descriptor for assessing vigilance and fatigue states in tasks requiring sustained attention.¹³ The Theta-Alpha-to-Beta Ratio (TA2BR) is calculated by summing the power of slow waves (theta and alpha) and dividing them by the power of fast waves (beta). Furthermore, advanced source localization methods, such as sLORETA, have identified the hyperactivity of the amygdala and the involvement of the dorsolateral prefrontal cortex as central to the reward-punishment processing loop.¹⁴ Finally, multivariate modelling in high-stress environments has shown that lower beta-band activation in the frontal cortex correlates with better situational performance and reasoning, suggesting that high performance is underpinned by greater neural efficiency and reduced cognitive load.¹⁵

Overall, in previous research studies, there have been extensive investigations into EEG spectral biomarkers and event-related potentials (ERPs) to understand cognitive control, impulsivity, and feedback processing. The studies show that frontal theta activity is a central mechanism for action monitoring, and the theta-to-beta ratio is a biomarker for self-regulation and inhibition, while neural markers such as Feedback-Related Negativity (FRN) and the P3 component have been identified as critical indices for identifying prediction errors and updating internal models. However, there was a clear research gap. Firstly, Most studies collapse behavioral data across entire experiments, failing to capture the trial-by-trial adaptation necessary to quantify an individual's innate capacity for cognitive re-evaluation. Secondly, while EEG biomarkers of conflict and adaptation are well established, they have not been systematically integrated into a reproducible, survey-free framework that captures neural receptiveness to belief updating as a cognitive state. Publicly available EEG datasets, particularly those involving cognitive conflict and feedback, remain underutilized for identifying cross-task neural signatures that precede persuasion or conceptual change. As a result, there is currently no standardized EEG-based metric that operationalizes when the brain enters a "persuasion-ready" or re-evaluation state. Thus, it was hypothesized that individuals who exhibit stronger frontal theta reactivity, adaptive theta-beta ratio modulation, and sustained post-conflict neural adjustment across trials demonstrate greater neural receptiveness to belief updating. By analyzing open-access EEG datasets and integrating these spectral and adaptation features into a novel Neural Re-evaluation Index (NRI), this project aims to address the gap by providing a reproducible, EEG-only marker of cognitive re-evaluation capacity that generalizes across tasks and datasets.

■ Methodology

Data Collection.

To acquire data for analysis, public datasets of EEG data were searched, and an appropriate dataset was chosen from an open-access repository of neuroimaging data in November 2023, from the study 'Investigating the cognitive conflict triggered by moral judgement of accidental harm: an event-related potentials study' (Flora Schwartz, Radouane El-Yagoubi, Julie Cayron, Pierre-Vincent Paubel, and Bastien Tremoliere, n.d.). The data set consists of electroencephalography (EEG) data recordings of 31 healthy participants analyzed using event-related potentials (ERPs). The data set was originally collected as part of a controlled laboratory research program using a third-party task (N400). Participants heard stories that involved morally sensitive situations with deliberate or unintentional harm and had to indicate whether a final word displayed, and a statement below was congruous with the story by pressing a response button. The EEG findings were collected continuously and then divided into event-related potentials (ERPs), which were time-locked to the presentation of the target word. It consists of raw EEG records, event markers, and metadata about subjects and experimental conditions. Trials were explicitly labeled as either intentional or accidental, and congruent or incongruent, and condition-wise ERP comparisons were possible.

Feature Engineering:

EEG processing was done using a pipeline in the Anaconda environment. The code compared the trial sequences to generate numerical features of different patients per event using ratios such as the theta-beta ratio. The theta-beta ratio (TBR) was calculated as the ratio of mean theta band power (4–7 Hz) to mean beta band power (16–31 Hz) for each epoch, and log-transformed for normalization before further analysis. The pipeline took data in BIDS structured EEG experimental data with sampling rates and metadata for events. Event onsets were identified based on the timestamp values that are stored in files in the BIDS events.tsv that contain the time when each stimulus was presented over the one-hour continuous EEG recording. These timestamps were used to segment the continuous EEG signal into epochs, segments of time of the signal surrounding an event. The raw EEG signals were band-pass filtered between 0.5 and 40 Hz to remove slow drifts and high-frequency noise. Ocular and movement-related artifacts were addressed using automated artifact rejection based on amplitude thresholds, and noisy epochs exceeding $\pm 100 \mu V$ were excluded from further analysis. These epochs were time-locked to a definition with a discrete pre-event and post-event baselines. The analysis was limited to frontal EEG channels - Fz, FC1, FC2, AF3, and AF4. Each of the epochs was converted from the time domain to the frequency domain to look at the spectral content, which was the breakdown of the EEG signal into its underlying frequency components. Mean power in theta and beta in the oscillatory activity.

Neural Re-evaluation Index Building:

NRI is a composite EEG index that is based on frontal theta modulation during cognitive conflict. In this case, frontal theta is usually linked to the detection of conflict and the recruitment of one's cognitive control, and the TBR corresponds to the balance between cognitive control and good temporal stability. Through their combination, and together in NRI, the NRI provides a physiological measure of a person's readiness to update their internal representations when confronted with information that is incongruent or conflicting. In this paradigm, 'conflict' was used to refer to semantically incongruent (REVAL_TRIGGER) target words (compared to congruent (BASELINE) trials) with the preceding moral scenario. The NRI was the measure of the difference in the neural activity of the REVAL_TRIGGER and the BASELINE trials. It measured the reactivity with the amount of frontal theta activity increased immediately after a conflicting target word was presented, and the regulation balance with changes in TBR. Theta activity correlated with conflict detection and control engagement, but the beta activity was correlated with continued interpretations or cognitive stability. The activation of the TBR was reflected in how the brain changes between incorporating new information and storing existing information. Thus, using the combination of frontal theta reactivity and theta-beta dynamics, the NRI was able to capture both rapid detection of conflict and the possible updating of an initial interpretation.

■ Results

Input Features Used For NRI:

Neural features based on post-target EEG activity were used to operationalize the NRI. This consisted of two neural features (frontal theta reactivity and TBR reactivity) for several time windows in aggregate at the participant level. Frontal theta reactivity (4-7 Hz) was used as an indicator of the cognitive control engagement during the re-evaluation triggers. Incongruent target words were linked to more frontal theta power than congruent trials. Frontal theta reactivity was defined as the difference between theta power during REVAL_TRIGGER and BASELINE trials. The difference was able to isolate the increase in neural activity that was specifically associated with conflict and not general processing. A greater increase in frontal theta reflected a greater involvement of conflict detection and control-related processing. Frontal theta reactivity and TBR reactivity were used as indices of shifts between control-related processing associated with theta activity and maintenance-related processing associated with beta activity during re-evaluation. Therefore, the ratio reflected the relative dominance of updating-oriented to more stability-oriented neural dynamics. The TBR reactivity was calculated as the difference between the REVAL_TRIGGER and BASELINE trials. A larger increase in TBR was suggestive of a relative shift away from maintenance-related processing and increased participation of control-related mechanisms related to potential belief updating. Post-target activity was analyzed within a series of different short time windows, with the time window 0.0-0.8s after target onset being further divided into

a series of smaller time intervals for distinguishing between early and later stages of neural modulation. The Theta and TBR values were both extracted separately for each window. Through the analysis of several windows, the index represents dynamic processing in the brain and not a single reaction. Due to differences in neural responses in individual trials, trial-level features were summarized into subject-level metrics. For every individual, the theta reactivity and TBR reactivity values were averaged between trials for each condition. This aggregation minimized variability over time and identified stable patterns of neural engagement. The number of trials was substantial for both the BASELINE and the REVAL_TRIGGER conditions, as shown in Figure 1.

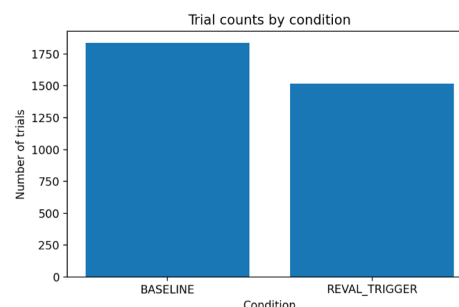


Figure 1: A bar graph of the number of trials for each condition. The x-axis is the quantity of the sum of the number of trials of the analysis (BASELINE and REVAL_TRIGGER), and the y-axis is the quantity of trials included. The blue bars are the aggregate trial count of the trials of all participants for each condition. The graph indicates a moderate imbalance in trial counts, with more trials in the BASELINE condition compared to the REVAL_TRIGGER condition.

Labelling Of The Re-evaluation Trigger:

Using predefined labels, which are specified in the BIDS event files of the dataset, the target events were systematically grouped into two conditions (BASELINE and REVAL_TRIGGER). It indicated whether each target trial was a congruent condition or an incongruent condition to the previous scenario. Those re-evaluation moments, including a semantic inconsistency between the scenario and the final target word, were REVAL_TRIGGER. If the following target word was incongruent, it did not match this established representation, and a measurable prediction error was created at the moment of word presentation. Therefore, REVAL_TRIGGER trials were the control trials in which re-evaluation was experimentally induced and measured in terms of neural differences, evidenced by the increased frontal theta and theta-beta reactivity (Figures 2,3). All condition assignments were made directly from the standardized metadata that was provided in the dataset by filtering events based on predefined condition labels specified in the dataset metadata, and can be reproduced using the same condition mapping, strengthening the reliability of the NRI construction process.

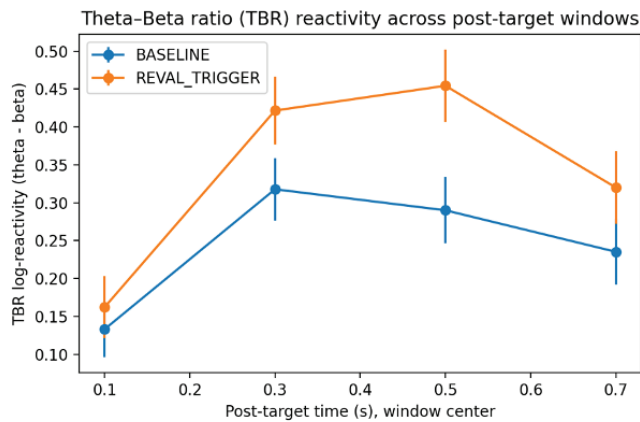


Figure 2: A line graph of Theta-Beta Ratio (TBR) reactivity across windows. Post-target time windows (0.1-0.7 s) are shown on the x-axis, and log TBR reactivity (theta - beta) is shown on the y-axis. The color blue is used to represent the BASELINE (congruent) trials, and the color orange is used to represent the REVALTRIGGER (incongruent) trials. Points indicate mean values across participants, error bars represent $\pm$ standard error. TBR reactivity is consistently higher in the REVAL_TRIGGER condition than in the BASELINE condition across all time windows, with the largest differences observed around 0.3–0.5 s.

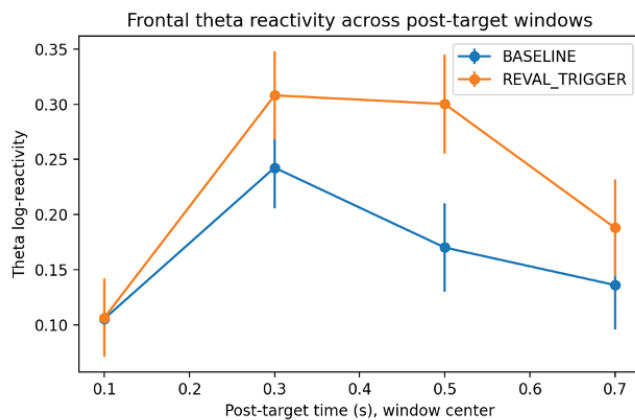


Figure 3: A line graph for Frontal theta reactivity over post target windows. The x-axis is post-target time windows (0.1 - 0.7 s); the y-axis is log frontal theta reactivity (4 to 7 Hz). The color blue stands for baseline (congruent) trials, and orange stands for REVALTRIGGER (incongruent) trials. Points indicate mean values across participants, error bars represent $\pm$ standard error. Frontal theta reactivity is higher in the REVAL_TRIGGER condition than in the BASELINE condition across most time windows, with the largest differences observed around 0.4–0.5 s.

Subject-level Aggregation:

For the subject-level aggregation, the mean frontal theta power and TBR values were computed for all the BASELINE trials and separately for all the REVALTRIGGER trials. This led to four main condition-specific averages for each subject: mean thetabaseline, mean thetatrigger, mean TBRbaseline, and mean TBRtrigger. The result of aggregation at this stage was that random trial-to-trial fluctuations were reduced and a more stable, condition-specific estimate was obtained for each participant. The values of Frontal theta modulation were the increase in frontal theta specifically related to the incongruent stimuli. The distribution of the scores of modulations at the subject level is shown in Figure 4. The score reflects a change in the regulatory balance in conflict and non-conflict condi-

tions. Z-score normalization was used between participants because these measures are on different numerical scales. The neural responses were compared based on what happened in the previous trial to see if conflict in one trial affected neural activity in the next trial. This sequence-based measure presents trial-by-trial adjustment in cognitive control following recent conflict.

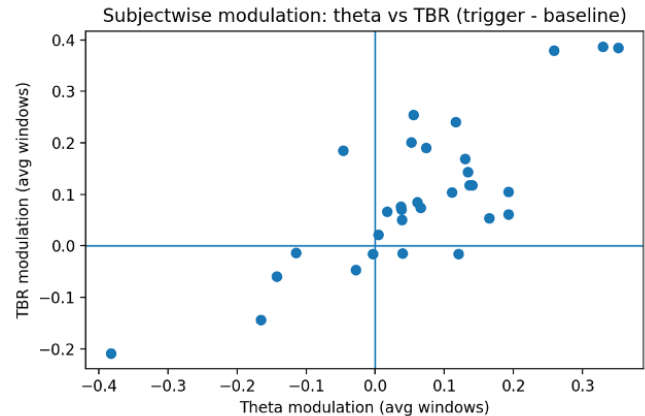


Figure 4: Subject-Level Modulation of Frontal Theta and Theta-Beta Ratio (TBR). The x-axis is the mean frontal theta modulation, and the y-axis is the mean TBR modulation (REVALTRIGGER, BASELINE), averaged on post-target windows. Each dot represents a single participant, and the reference lines at zero represent no modulation relative to baseline; positive values indicate increased activity during REVALTRIGGER trials. The figure shows positive modulation in both theta and TBR, with a general positive association between the two measures.

Normalization, scaling, and the final NRI formula:

The frontal theta modulation and TBR modulation were measured on different numerical scales, which led to each subject-level component being standardized before combination. Z-score normalization was performed across participants to ensure comparability of neural measures on a common scale. For each of these features, the group mean was subtracted from each participant's value and divided by the group standard deviation. After normalization, the NRI composite score was computed by synthesizing the average of standardized frontal theta modulation, standardized TBR modulation, and standardized adaptation component to generate one index value for each participant. This led to less reliance on any single feature, which portrayed a more general pattern of conflict-related neural involvement. The formula was kept deliberately simple so as not to lose conceptual clarity. The index was kept simple to grasp and associated with the underlying neural features (by not having complex weighting or multi-step transformations). NRI ensured that differences in neural re-evaluation readiness are examined quantitatively and proportionally, consistent with the interpretation of the index, and with the graded nature of neural responses.

Validation of NRI:

Internal validation of the NRI was done by testing whether the index was grounded in conflict processing. The incongruent trials would elicit higher theta and TBR values for the incongruent trials than the congruent trials. This pattern was indicative of a higher level of engagement of neural systems

that are involved in conflict detection and control allocation. The finding of the expected increase in the REVALTRIGGER conditions supported the construct validity of the NRI. It showed that the neural features that were used to construct the index were sensitive to experimentally caused conflict. Computational validation of the NRI was performed by means of a Leave-One-Subject-Out (LOSO) decoding strategy. A logistic regression classifier with default L2 regularization was used for decoding, implemented using standard machine learning libraries in Python. The classifier resulted in the Area Under the Curve (AUC) value of 0.543 as shown in Figure 6. A total of theta, beta, and TBR features extracted across multiple post-target time windows were used as input to the classifier. Feature importance analysis gives information about the features that contributed most to classification as part of the neural components. As can be seen in the feature coefficient plot, LOSO folds were associated with theta and TBR features in early-to-mid post-target windows, theta0p40p6 and tbr0p4_0p6. These windows are associated with the period in which the strongest group-level modulation was observed, indicating that neural activity during the 0.4-0.6 s period contributed most to the discrimination of whether or not conflict occurred in comparison to the baseline trials. Although the classification performance across all cases was modest, the robustness of the contribution of these features suggests that there is something in the conflict-related neural modulation that can be generalized across individuals. A positive association between NRI and RT cost suggested that people who show more neural engagement of conflict were also more prone to a behavioral slowing during re-evaluation triggers. Behavioral effects were not always found to be consistent across paradigms, and response timing was observed. In tasks where the accuracy was high or the timing of response was a constraint, RT differences may be small even when neural differences were robust. Therefore, a poor or non-existent behavioral correlation does not necessarily invalidate the NRI.

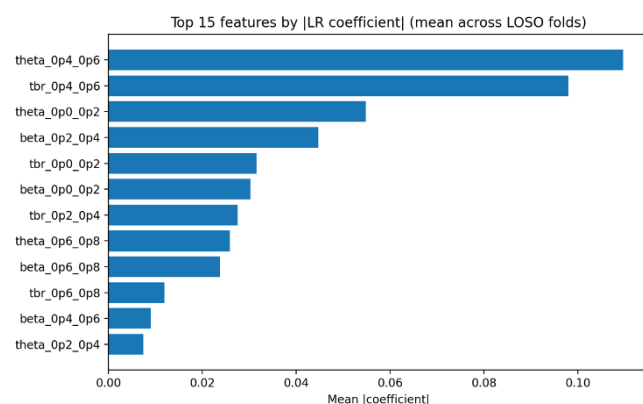


Figure 5: Top features ranked by Mean Absolute Logistic Regression Coefficient (LOSO). The x-axis represents the average (across Leave-One-Subject-Out, LOSO) of the absolute value of logistic regression coefficients. The y-axis is the list of top features in terms of importance, taken from specific time windows after targeting. Theta and TBR features, particularly in later time windows (e.g., 0.4–0.6 s), show the highest coefficients and therefore contribute most strongly to classification performance.

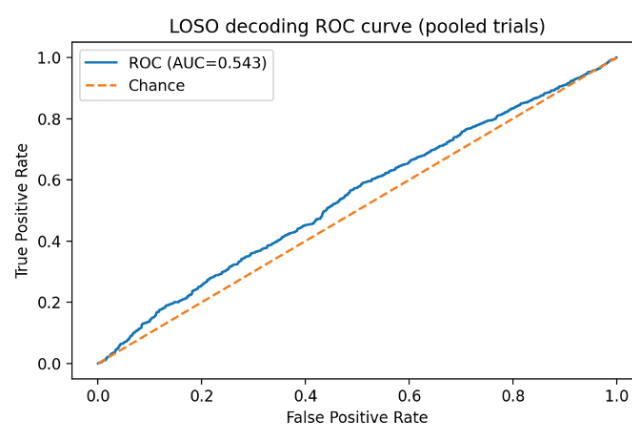


Figure 6: Leave-One-Subject-Out (LOSO) decoding Receiver Operating Characteristic (ROC) curve. On the x-axis is the false positive rate, and on the y-axis is the true positive rate. The solid blue line shows the classifier performance (AUC=0.543) and the dashed orange line shows the chance level performance (AUC=0.50). The ROC curve is a grouped curve of the trial predictions based on LOSO folds. The classifier performs only slightly above chance (AUC = 0.543), indicating limited discriminative ability between conditions.

Interpretation of NRI In The Context of The Research Question:

NRI can be interpreted as a neural indicator of cognitive re-evaluation capacity, which reflects the degree to which an individual's brain recruits conflict-related control mechanisms when faced with information that contradicts prior expectations, with a high NRI value indicating a higher degree of neural modulation during conflict. This indicates a more intensive activation of systems that are responsible for discrepancy monitoring and internal representation adjustment. The NRI, therefore, is a physiological index of readiness to change existing representations according to new information. It also allows for millisecond-level temporal resolution, which enables researchers to see neural responses at the time when conflicting information was encountered. These responses are often made before a conscious decision is made or verbally expressed. As a result, the NRI may be able to capture states related to pre-conscious readiness, that is, early neural indications that a person is allocating cognitive resources to processing and perhaps updating information. The NRI is an objective measure of belief revision efforts, and therefore could indicate whether or not a correction has been successful in getting the brain's regulatory circuitry to kick in through TBR modulation or if the correction has been turned away at a pre-conscious level.

■ Discussion

This project constructed a neural metric, the NRI, designed to quantify how strongly individuals engage neural mechanisms associated with conflict detection and cognitive re-evaluation. A publicly available EEG dataset involving moral scenarios followed by congruent or incongruent target words was utilized. By using the metadata and markers in the dataset, the frontal theta activity and the theta-beta ratio were extracted as neural markers of conflict processing. The calculated differences in these signals between incongruent and congruent trials were used to construct the NRI, which was then evaluat-

ed using statistical comparisons and cross-validated decoding analyses. The results confirmed that incongruent stimuli elicit a significantly higher theta and TBR value at the group level.

The methodology of the contracted NRI aligns with recent advancements in technique-focused EEG research. A previous study demonstrated that frontal-midline theta oscillations serve as a real-time marker of cognitive control during the resolution of conflicting interpretations.¹⁶ This project utilized theta-band activity to capture the brain's response to semantic mismatch. Furthermore, the usage of the theta-beta ratio as a core component of the NRI mirrors another study, which found that frequency ratios such as the TAR and TA2BR are superior descriptors of cognitive effort and vigilance compared to single-frequency bands alone.¹³ By adopting a trial-by-trial aggregation approach rather than collapsing data across the entire experiment, this project follows the dynamic statistical modelling proposed by a research study on frontal alpha asymmetry, predicting subsequent social decision-making, allowing for a more nuanced observation of how neural states shift in response to specific feedback.¹⁷

In terms of results, the NRI modulation patterns support the "functional dissociation" theory observed in prior decision-making studies. As explored in a previous study, there is a documented split where theta activity is linked to impulsive choice and beta activity to cognitive control.¹⁸ Similarly, this paper's findings suggest that an increased TBR occurs during moments requiring the suppression of an initial mental model. However, some studies have found that individual traits like social dominance significantly modulate the Feedback-Related Negativity (FRN) in response to uncertainty; the NRI focuses on a broader, task-agnostic indicator of "re-evaluation readiness."¹⁰

The primary limitation of the project is the relatively small sample size of 31, which may limit statistical power and the generalizability of the findings. This could improve the classifier, which showed only a modest discriminative performance, suggesting that the current feature set captures conflict-related neural signals only partially. As a future scope of this study, additional studies would be examined for the behavioral validity of the NRI by correlating NRI values with reaction time (RT) costs and accuracy differences between conflict and baseline trials. The framework would also be tested on established paradigms such as the Stroop and Flanker tasks to evaluate if the NRI reflects a task-agnostic measure of conflict processing. Further validation across paradigms will help determine whether the index can predict adaptive trust calibration in human and AI interaction contexts.

■ Conclusion

In conclusion, this project addresses a critical research gap by capturing dynamic and trial-by-trial neural signatures of cognitive re-evaluation by moving beyond static mode. A basic structure for an objective, persuasion-ready metric was achieved in the combination of frontal theta reactivity and TBR modulation into a combined index. The low performance of 0.543 obtained by the classifier is reflective of how hard it is to identify

belief-updating states across individuals. A steady modulation in the neural activity during conflict triggers suggests that the NRI acquired meaningful involvement of cognitive control processes. The utility of this study is that it has developed a reproducible EEG-only framework, which avoids the limitations of traditional self-report surveys. The NRI provides one way of quantifying "re-evaluation readiness" on a pre-conscious level. This approach provides a process-level index of how people identify and deal with conflicting information, which may have applications in the literature of conceptual change, misinformation resistance, and adaptive human-AI interaction. Overall, the NRI provides a reproducible and objective EEG-based framework for quantifying neural readiness for cognitive re-evaluation, offering potential applications in understanding adaptive decision-making and resistance to misinformation.

■ Acknowledgments

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The Future of Medicine: CAR-T Cell Therapy, CRISPR, And Gene Editing for Glioblastoma

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ABSTRACT: Glioblastoma Multiforme is the most aggressive and fatal malignant brain cancer. Despite conventional treatment (surgery, radiation, and chemotherapy), the median survival rate remains less than 18 months. Conventional therapies have significant limitations due to GBM's inherent heterogeneity and its micro-environment's immunosuppression; therefore, new, more durable, targeted therapeutic approaches need to be developed to increase patient survival. Current evidence indicates that utilizing CRISPR-Cas9 gene editing in combination with chimeric antigen receptor (CAR)-T cell therapy will provide a new approach to overcoming several of the current barriers to effective GBM immunotherapy. CAR-T cells have been developed using various GBM-specific antigens (EGFRvIII, IL13R α 2, HER2, GD2, and EphA2). CAR-T cells directed at these antigens have demonstrated some level of clinical response as well as acceptable safety profiles, but generally lack long-term efficacy. Utilizing CRISPR-engineered CAR-T cells that incorporate deletion of inhibitory receptors (such as PD-1 and TIGIT) and integrate stable CAR constructs demonstrates increased persistence, immune activation, and tumor killing capabilities in all pre-clinical models evaluated. Therefore, integrating CRISPR-based genetic modification with CAR-T cell therapy has the potential to develop more personalized and durable immunotherapies for GBM, which could revolutionize the treatment of what is currently considered one of the most treatment-resistant malignancies.

KEYWORDS: Disease Treatment and Therapies, Glioblastoma, CAR-T Cells, CRISPR-Cas9, Immunotherapy.

■ Introduction

Glioblastoma multiforme (GBM) remains one of the most aggressive and treatment-resistant primary brain tumors, with median survival rarely exceeding 18 months despite maximal surgical resection, radiotherapy, and temozolomide chemotherapy.¹ Current therapeutic strategies are largely palliative, failing to eradicate infiltrative tumor cells or prevent recurrence due to GBM's high genetic heterogeneity, antigenic variability, and profoundly immunosuppressive microenvironment.² Immunotherapies such as checkpoint inhibitors and cancer vaccines have demonstrated limited efficacy in GBM, primarily because the blood-brain barrier, as mentioned in Table 2, restricts immune cell access, and the tumor's adaptive resistance mechanisms enable immune evasion and limit treatment options.^{3,4} Consequently, there is an urgent need for therapeutic approaches that can specifically and effectively target tumor-associated antigens while overcoming immune suppression within the central nervous system.⁵

Immunotherapy has been proposed as a new generation of treatments for GBM that can overcome the limitations of traditional treatments. Immunotherapy involves using genetically engineered T lymphocytes that express chimeric antigen receptors (CAR).^{6,7} CAR-T cells are engineered to specifically target and kill tumor cells that express particular antigens such as EGFRvIII, IL13R α 2, HER2, GD2, and EphA2.^{8,9} Clinical trials have shown that CAR-T therapy can be safely administered to patients with GBM and, in some cases, cause measurable reductions in tumor size.^{9,10} However, because of the heterogeneity of tumor antigens expressed by GBM cells,

the potential for tumor cells to develop mechanisms to avoid destruction by the CAR-T cells, and the immunosuppressive environment of the tumor, CAR-T cell-mediated anti-tumor effects are usually transient and do not lead to durable therapeutic benefits.^{1,3,11}

In some Preclinical studies, scientists have used the CRISPR/Cas9 gene-editing system to prolong the duration of CAR-T cell activity, more effectively recognize tumor antigens, be more resistant to immune suppression, and persist longer in the body, suggesting a possibility of longer-lasting and more effective anti-tumor effects with less toxicity.^{12,13}

Considering the challenges of treating GBM with current therapies and the promise of using genetic engineering to create more effective and targeted treatments, the integration of the CRISPR system into CAR-T cell therapy is a timely and promising method of addressing many of the challenges associated with treating GBM. Therefore, the purpose of this study is to integrate the knowledge currently available regarding CAR-T and CRISPR-based therapies, assess the translational implications of these therapies, and explore the possible combination of these two therapies to provide new and innovative treatment options for glioblastoma, a cancer with one of the worst prognoses in human medicine. This literature review brings together many of the latest clinical trials of CAR-T cell therapy. Most of them include phase 1 trials that aim to develop long-lasting, individually designed treatments for Patients with Glioblastoma and are significant contributions to Translational Medicine and Cancer Immunotherapy.

■ Methods

This study employed a narrative literature review design rather than an experimental or in-field study. The literature review method was chosen to synthesize information from previously published peer-reviewed research, clinical trial data, and institutional reports. No laboratory experiments, surveys, or human subjects were directly involved. The study did not require any ethical clearance, as it involved secondary data sources exclusively.

Relevant studies were identified and collected from credible online databases between 2010 and 2025. Databases searched included PubMed, Google Scholar, ClinicalTrials.gov, and the National Institutes of Health (NIH) repositories. The key search terms used were: “glioblastoma,” “standard treatment,” “CAR-T therapy,” “CRISPR-Cas9,” “GBM tumor antigens,” and “immunotherapy in GBM.” Studies were filtered to include those that provided data on treatment mechanisms, therapeutic efficacy, and translational relevance to clinical applications.

Primary sources for this review included landmark clinical and epidemiological studies, such as the Stupp regimen trial on chemoradiation in GBM and the CBTRUS report on glioblastoma incidence and survival data. Clinical trial data from ClinicalTrials.gov focusing on CAR-T therapies against EGFRvIII, IL13Rα2, HER2, GD2, and EphA2 antigens were also reviewed. Secondary sources included translational and molecular studies that explored the role of CRISPR-Cas9 in enhancing CAR-T cell safety, persistence, and specificity.

All selected studies were reviewed for methodological quality, relevance, and results. Data on therapeutic efficacy, antigen targets, clinical outcomes, and genetic editing strategies were categorized into thematic areas—namely: glioblastoma biology and standard treatments, CAR-T therapy design and results, CRISPR-based modifications, and clinical translation and challenges. Findings were compared and summarized to highlight the collective progress and limitations within the field.^{8,14,15}

No physical tools, laboratory materials, or experimental equipment were used in this study. All information was obtained through online academic and clinical databases. Since this research relied solely on existing published literature and publicly available data, no ethical review or participant consent was required.

Prevalence and Epidemiological Profile of Glioblastoma:

Glioblastoma (GBM) is the most common malignant primary brain tumor in adults. Globally, it affects about 3–5 people per 100,000 per year, making it a relatively rare cancer, but the most frequent among aggressive brain tumors. It represents about 15% of all brain tumors and nearly 50% of malignant gliomas. Men are slightly more likely to develop GBM than women. Most cases are diagnosed in people aged 45–70, though it can occur at younger ages as well.¹⁴

GBM is a WHO Grade IV glioma with necrosis, microvascular proliferation, and several genetic alterations, including TERT promoter mutation (a genetic alteration that increases telomerase expression, enabling tumor cells to maintain telo-

mere length and achieve uncontrolled growth and survival),¹⁶ EGFR amplification (an abnormal increase in the number of copies of the EGFR gene), which drives uncontrolled cell signaling, proliferation, and tumor growth),¹⁷ chromosome 7 gain/10 loss (+7/-10), and alterations in critical pathways are RTK/RAS/PI3K, p53, and RB. GBM is strongly resistant to conventional therapy because of heterogeneity of the tumor and an immunosuppressive tumor microenvironment, hence the justification for targeted immunotherapy such as CAR-T.^{17,18}

Genetic Mutations :

Many genetic mutations cause aggressive behavior in GBM.

TERT promoter mutations: Increasing the TERT activity due to mutations of the TERT promoter allows cancer cells to divide forever. Cancer cells become capable of dividing continually because the mutations increase telomerase activity of an enzyme called telomerase, which adds repetitive DNA sequences to the telomere's chromosome ends and keeps them from shortening. Telomerase prevents the normal process of aging of cells by preserving their telomere lengths, thereby allowing cancer cells to divide infinitely, beyond the bounds of typical cell division.¹⁵

EGFR mutations or amplifications: cause increased cell proliferation and survival, thus causing tumors to grow more quickly.

In many cases of GBM, the EGFR gene is amplified or mutated. For example, a particular type of EGFR mutation, EGFRvIII, causes a truncated receptor that signals continually without the need for binding with an EGF ligand, promoting uncontrolled cell proliferation, angiogenesis, and cell survival.⁸

Abnormalities of Chromosomes (+7/-10): Both gain of chromosome 7 and loss of chromosome 10 occur commonly in GBMs, and both are involved in malignancy.¹⁹

Loss of chromosome 10 and gain of chromosome 7 are common characteristics of GBM. Loss of chromosome 10 removes tumor-suppressing genes (e.g., PTEN) while gaining chromosome 7 adds oncogenes (e.g., EGFR), both of which result in the shift toward cancer.²³

Disrupted signaling pathways: Many of the major signaling pathways involved in controlling cell growth and cell death, including the RTK/RAS/PI3K, p53, and RB pathways, are frequently altered in GBM, resulting in uncontrolled growth and failure to undergo programmed cell death.²⁰

RTK/RAS/PI3K pathway: Activation of the RTK/RAS/PI3K pathway through mutations of EGFR or PDGFRA results in activation of pathways that promote cell survival and proliferation.^{20,21}

p53 pathway: TP53 normally regulates cell death and DNA repair; when it is mutated, the ability of damaged cells to survive is enabled.²¹

RB pathway: The absence of Rb1 removes a critical regulatory point in the RB pathway that controls the cell cycle and allows for unregulated cell division.¹⁹

Other mutations: Other mutations, such as those in PTEN or IDH1/2, may influence prognosis and responsiveness to chemotherapy.

PTEN deletion: Inactivates a key inhibitor of the PI3K/AKT pathway, thus enhancing tumor growth and treatment resistance.

IDH1/2 mutations: IDH1/2 mutations are seen in approximately 50% of secondary GBMs, but less than 5% of primary GBMs. Secondary GBM patients who have these mutations may have a better prognosis and may respond to some types of chemotherapy.²² All of these genetic alterations contribute to the uncontrolled growth of GBM and are the reason that targeted therapies such as CAR-T cells are of interest.

Standard Treatments for GBM:

Glioblastoma (GBM) is highly aggressive and typically involves refractoriness to conventional treatment, requiring multimodal therapy:

Surgery: Cytoreduction by maximal safe resection removes much of the tumor and improves survival. Studies have shown that a maximal safe resection can significantly extend the median survival for certain patients. For example, some cohorts have shown an average survival of 37.3 months for patients who underwent aggressive resection, compared to 16.5 months for those with more modest resections.²³

Radiation + Temozolomide (Stupp Regimen): Studies show that the combination of radiation and temozolomide will give you a much better chance of survival than either treatment alone. The results of a major study by EORTC (European Organization for Research and Treatment of Cancer) and NCIC (National Clinical Trials Group).²⁴ This showed that the median overall survival time with this combination was 14.6 months and 12.1 months when using radiation alone. Also, the researchers found that patients who received the combination therapy were twice as likely to be alive after two years (26.5%) as those receiving radiation alone (10.4%). Additionally, studies indicate that if your tumor has the MGMT promoter methylation biomarker, there may be a better response to temozolomide chemotherapy; therefore, identifying this biomarker could provide useful information regarding which chemotherapy to use.²⁵

Tumor Treating Fields (TTFs): There is evidence indicating that the addition of TTFs to the Stupp regimen resulted in a statistically significant increase in survival time. The EF-14 study demonstrated that adding TTFs to the Stupp regimen increased the median overall survival time from 16.0 months to 20.9 months. Additionally, the study found that the addition of TTFs to the Stupp regimen resulted in significant improvements in long-term survival rates. Specifically, the two-year survival rate was increased from 30% to 43%, and the five-year survival rate was increased from 5% to 13%.²⁶

Bevacizumab: A VEGF-targeting therapy that manages symptoms and edema. For newly diagnosed glioblastoma, two large Phase 3 trials showed that bevacizumab did not improve overall survival when added to standard therapy.^{27,28} However, in patients with recurrent glioblastoma, it is a standard treatment that has demonstrated a significant effect on progression-free survival and response rates. For these patients, clinical data have shown a 6-month progression-free survival rate of approximately 42.6%.^{22,28}

These therapies, in general, are capable of managing tumor growth and symptoms temporarily, though GBM is still largely an incurable disease overall; targeted treatments like CAR-T cell therapy are needed.

Table 1: Summary of Standard Treatments for GBM.

Treatment	Mechanism of Action	Survival / Response Benefit	Notes/Limitations
Surgery (maximal resection)	Physical removal of as much tumor mass as safely possible.	Median survival up to 37.3 mo vs. 16.5 mo. ²³	Improves outcomes but cannot remove all infiltrative cells; tumor recurrence is common.
Radiation + Temozolomide (Stupp)	RT damages DNA; Temozolomide alkylates DNA, triggering apoptosis in tumor cells.	Median OS 14.6 mo vs. 12.1 mo with RT alone; 2-yr OS 26.5%. ²⁴	Standard of care; effectiveness is higher with MGMT methylation; resistance often develops
Tumor Treating Fields (TTF)	Alternating electric fields disrupt mitotic spindle formation, blocking cell division.	Median OS 20.9 mo vs. 16.0 mo; 5-yr OS 13% vs. 5%. ²⁶	Non-invasive and improves long-term survival; requires continuous device use, which affects compliance.
Bevacizumab (recurrent GBM)	Monoclonal antibody against VEGF → reduces angiogenesis and tumor edema.	6-mo PFS 42.6%. ²⁹	Effective for symptom relief and edema; no proven OS benefit in newly diagnosed GBM

The table illustrates that all of the established therapies will only marginally prolong life expectancy, but there are no established cures for Glioblastoma Multiforme (GBM). The table points out the shortcomings of traditional therapies and the need for the development of new therapeutic modalities that are both targeted and longer lasting than traditional therapies, i.e., immunotherapies.

Although the standard treatments - surgery, radiation therapy with temozolomide, Tumor Treating Fields, and bevacizumab for GBM- can extend survival and enhance quality of life, their advantages are still restricted. Surgery provides the greatest survival advantage when maximal safe resection is achievable. Even with the Stupp regimen, the median survival is only 16 months, and the five-year survival rate is around 6–7%.¹⁴ This is also mentioned in Table 1, where it shows multiple differences in the survival median for each of the different kinds of treatments, and the highest amount of survival is 37.3 months with surgery, and the lowest is 6 months with Bevacizumab, a treatment used for recurrent GMB. Although add-on strategies like bevacizumab and TTFs offer slight increases in survival or symptom control, overall results show the urgent need for more potent and focused treatments like CAR-T cell therapy.

Key Challenges in GBM Treatment:

GBM has a number of inherent challenges that reduce the efficacy of available treatments. These difficulties are caused by the intricate biology of the tumor as well as how it interacts with the protective environment of the brain. Developing more successful treatment plans requires an understanding of these obstacles. The main difficulties in treating GBM are enumerated in Table 2, along with how they affect the results of therapy.

Table 2: Key Challenges in the Treatment of GBM.

Challenge	Description	Example / Impact
Tumor heterogeneity	Cells within the same tumor can have different genetic and molecular profiles, which makes it hard for a single therapy to target all cancer cells effectively. ³⁰	EGFR amplification in one region, PTEN loss in another → therapy resistance. ³¹
Immunosuppressive microenvironment	GBM can suppress immune activity in its surroundings, reducing the effectiveness of immunotherapies. ²	PD-L1 expression inhibits T-cell activity, lowering immunotherapy efficacy. ⁵
Blood-brain barrier (BBB)	This protective barrier prevents many drugs from reaching the tumor, limiting treatment options. ⁴	Temozolomide crosses the BBB, but most monoclonal antibodies cannot. ³²
Infiltrative growth	GBM cells invade surrounding brain tissue, making complete surgical removal nearly impossible. ³³	Even after maximal surgery, recurrence occurs within ~2 cm of the margin. ³³

The table summarizes the major structural and biological barriers to treatment success in glioblastoma multiforme. Together, these interconnected barriers illustrate why current therapies are usually ineffective, as tumor heterogeneity promotes therapeutic resistance while the blood-brain barrier and immunosuppressive tumor microenvironment limit both drug delivery and immune-mediated tumor destruction. Therefore, these challenges reinforce the necessity for the development of novel methods to treat GBM, including CAR-T cell therapy and CRISPR-based approaches.

New Treatment Options:

Immunotherapy, including CAR-T cell therapy:

In comparison to conventional treatments, immunotherapies for glioblastoma multiforme (GBM) show promise of better results. The principle of immunotherapy is to utilize a person's immune system to specifically identify and destroy the malignant cells that comprise a tumor. Immunotherapies currently under investigation for the treatment of GBM include dendritic cell (DC) vaccines, checkpoint inhibitors, and CAR-T cell therapy.

CAR-T-cell therapy represents a paradigmatic example of how an immunotherapeutic strategy can be used to design and engineer a patient's T-cells to specifically identify and destroy malignant cells. Modifying T-cells achieves this, so they will produce a chimeric antigen receptor (CAR), which allows them to recognize and kill tumor cells expressing a specific protein marker(s) on the surface of those cells. Researchers are now investigating whether or not this therapeutic approach can also be applied to the treatment of solid tumors, including GBM. CAR-T cells are genetically engineered T-lymphocytes designed to produce chimeric antigen receptors (CARs), which allow them to recognize and kill cancer cells displaying a specific protein(s) on their surface. In order to administer CAR-T cell therapy, the patient's T-cells are removed from the patient, then altered in a laboratory to recognize a particular antigen expressed on the surface of the tumor cells, and finally, the T-cells are returned to the patient to assist in destroying the cancer cells.¹³

CAR-T cell therapy has been studied in addition to dendritic cell vaccine-based treatments and checkpoint inhibitors, as other forms of immunotherapy, which could potentially provide patients with alternative treatment options.

Checkpoint inhibitors function by blocking proteins (such as CTLA-4 and PD-1/PD-L1) that inhibit the activation of T-cells, as illustrated in Figure 1. When administered, checkpoint inhibitors allow T-cells in the patient's body to travel to the tumor more easily and kill the tumor cells. Although checkpoint inhibitors have shown great promise in the treatment of some cancers, the low expression of tumor-associated antigens and high degree of immunosuppression found in the GBM tumor microenvironment have restricted the effectiveness of checkpoint inhibitors in treating GBM.^{34,35}

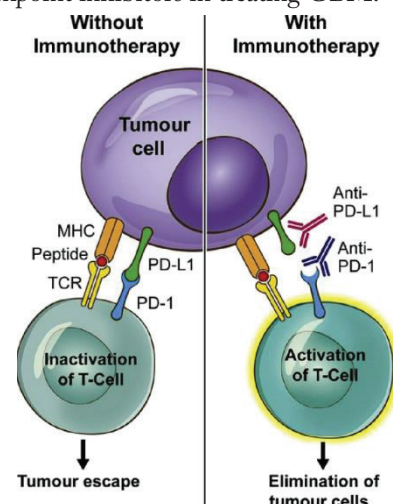


Figure 1: Visual representation of Checkpoint inhibitors. Genetically engineering T-cells with an antigen receptor is called CAR-T therapy; T-cells are taken from the blood of patients and put through a process of genetic modification in a laboratory using a viral vector to insert a gene that codes for a chimeric antigen receptor (CAR) on these T-cells. The chimeric antigen receptor allows the engineered T-cells to bind to a specific protein located on cancer cells. Once the CAR T-cells have been multiplied in the laboratory, they are injected back into the patient, where the CAR T-cells will continue to look for and kill cancer cells as they bind to their target antigen and induce apoptosis in the cancer cells.³³

DC (dendritic cell) vaccines are to activate the body's immune system through exposure of dendritic cells to specific antigens present on cancerous cells (i.e., EGFRviii), as shown in Figure 2. As such, the hope is to create a targeted T-cell response to GBM without attacking healthy brain tissue. While the approach has proven successful for GBM patients on Provenge, the use of DC-based cancer vaccines is considered safe; they have failed to provide significant clinical benefits to patients, with only 5-15% of patients experiencing an objective immune response. It is possible that many of these failures in the efficacy of cancer vaccines resulted from the immunosuppressive mechanisms acting on the antitumor T cell responses; therefore, these factors represent a common rate-limiting step in most clinical trials and limit the overall clinical benefit of cancer vaccines.³⁶

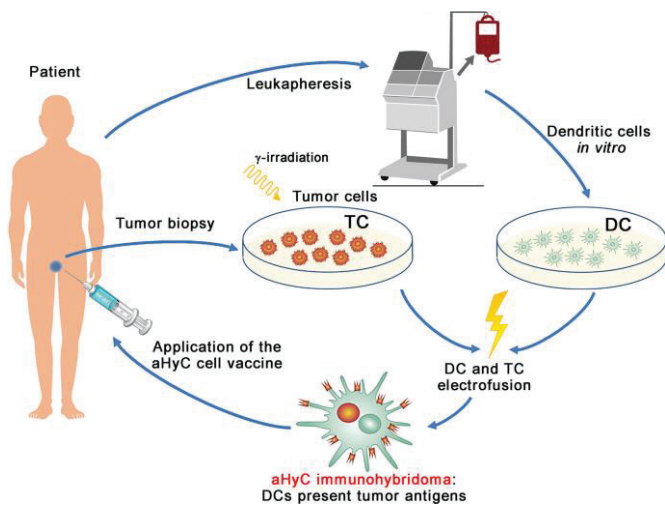


Figure 2: The hybrid personalized cancer vaccine involves preparing two different types of cells, namely tumor cells (from a biopsy) and dendritic cells (obtained through a simple blood draw), which are each treated separately and then combined through the use of electrical shock. The combination of the tumor's antigens with the dendritic cell's capacity for activating the immune system creates a "hybrid" vaccine. This vaccine is then returned to the patient as an injection, and causes the T-cells in the patient to recognize a wide variety of their own tumor markers, which results in a strong and targeted immune response against the patient's cancer. Visual representation of DC (dendritic cell) vaccines, adapted from Reference 37

Checkpoint inhibitor therapies and DC vaccine-based therapy often fail in glioma. This is because of the tumor's immunosuppressive microenvironment, as mentioned in Table 2, which limits the effectiveness of the immune activation, and glioma cells frequently lack strong neoantigen expression needed for robust T-cell responses, resulting in poor efficacy.³⁴ CAR-T remains the most heavily pursued immunotherapy in GBM as it can directly target tumor antigens and get around some of the limitations of other immune approaches.⁹

CAR-T Concept and Generation Using CRISPR Technology:

The CAR-T cell treatment process includes removing a patient's T-cells via leukapheresis and genetically modifying those cells so they can detect and kill cancer cells that contain the same proteins on their surfaces; proteins such as the EGFRvIII protein that some glioblastomas will express. Genetically modified T-cells are made by adding a Chimeric Antigen Receptor (CAR) to the T-cells' membrane. The main concept of CAR is to merge the native antigen recognition capacity of BCR (B cell receptor or antibody molecule) and the T cell activation capacity of TCR (T cell receptor). It thus bypasses the 'MHC-restriction' for recognition and killing of tumors by T cells. More in-depth into this idea, the CAR is made from an engineered receptor that contains both an antibody-derived recognition domain (the most common being a single-chain variable fragment, or scFv) and a T-cell signaling domain. CARs consist of three primary parts: an antigen-binding domain that sits outside the cell and binds to antigens on the surface of tumor cells; a transmembrane domain that holds the CAR in place; and an intracellular signaling region containing the CD3ζ chain and possibly one or two co-stimulatory molecules (CD28 or 4-1BB) to enhance T-cell activation, persistence, and ability to kill tumor

cells.¹⁰ After genetic modification, CAR-T cells are cultured *in vitro* and returned to the patient; usually after a course of lymphodepleting chemotherapy to allow the CAR-T cells to engraft and persist. Once returned to the patient, the CAR-T cells target and destroy tumor cells, produce cytokines, and attract other immune responses. Despite its potential, however, multiple barriers limit CAR-T cell treatment effectiveness in Glioblastoma Multiforme (GBM); among them are tumor heterogeneity resulting in antigen loss/escape, an immunosuppressive tumor microenvironment, limited T-cell penetration to tumors due to the presence of the blood-brain barrier, and the potential toxicity of CAR-T cell therapies such as cytokine release syndrome (CRS) and neurotoxicity, as summarized in Figure 3.³⁸

Genome engineering advancements using CRISPR-Cas9 have improved CAR-T cell development and deployment to increase the efficacy and safety of CAR-T cells. By utilizing CRISPR to remove the inhibitory receptors PD-1 and/or TIGIT from CAR-T cells expressing an anti-EGFRvIII construct in glioblastoma models, researchers were able to demonstrate that CAR-T cells exhibited greater tumor killing capabilities and increased survival duration than did previous generations of CAR-T cells.¹¹ Additional enhancements include employing CRISPR to integrate the CAR transgene into "safe harbors" of the genome where it will be stably expressed, developing "universal" allogeneic CAR-T cells that lack TRAC and B2M genes to minimize the risk of graft-versus-host disease and immune rejection, and researching methods to generate CAR-T cells *in vivo* to decrease the need for large-scale manufacturing of CAR-T cells and associated costs. In total, the CRISPR-related advances above are expected to improve the persistence, uniformity, and overall effectiveness of CAR-T cells in treating glioblastoma multiforme.⁴⁰

Recently, improvements in genome engineering technologies, specifically CRISPR-Cas9, have been utilized to enhance the design of CAR-T cells and improve their efficacy and safety. For example, CRISPR-Cas9 was used to ablate the inhibitory receptors PD-1 and/or TIGIT from CAR-T cells expressing an anti-EGFRvIII construct in preclinical glioblastoma models, which resulted in significantly enhanced tumor killing capability and prolonged survival upon intracerebral delivery. (34)³⁹ Additional modifications include knocking in CAR constructs to more controlled genomic locations and generating "universal" allogeneic CAR-T cells through disruption of T-cell receptor (TRAC) and beta2-microglobulin (B2M) to diminish the risk of graft-versus-host and rejection. As such, the CRISPR-engineered CAR-T cell designs described herein should ultimately provide superior T cell persistence, uniform CAR expression, and may enable future *in vivo* generation of CAR-T cells without extensive ex vivo expansion, thus lowering the cost and time required for CAR-T cell manufacture.⁴⁰

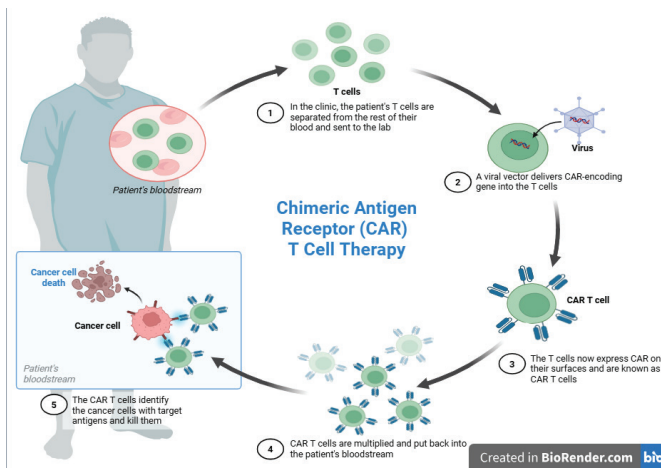


Figure 3: CAR-T Cell Therapy - Production and clinical use. This graph shows the full cycle of a CAR-T cell from its production to its implementation back into the body. Tumors can shut down T-cells by binding their PD-L1 to the T-cell's PD-1 receptor and essentially shut down the T-cells' ability to find and kill the tumors. Checkpoint Inhibitors are a class of immunotherapies that target PD-1 or PD-L1 and stop the interaction with the "off" switch for the T-cell, so they do not go into a dormant state and can continue to identify, target, and destroy cancer cells that may have otherwise gone undetected by the body's immune system. This graph was built on BioRender.

CAR-T Cells in Glioblastoma: Mechanism and Clinical Insights:

CAR-T cell therapy relies on identifying tumor-specific antigens that can distinguish malignant cells from normal brain tissue. These antigens serve as molecular "flags" on the tumor surface, guiding CAR-T cells to recognize and destroy cancer cells. In glioblastoma (GBM), several key antigens have been investigated as therapeutic targets due to their overexpression, tumor specificity, and association with aggressive disease biology (Table 3).^{10,41}

Table 3: Antigen Targets in GBM.

Antigen	Description and Clinical Insight	Current Clinical Status
EGFRvIII	A mutant form of the epidermal growth factor receptor is present in ~25-30% of GBMs. It promotes uncontrolled cell proliferation and enhances tumor survival, making it an attractive but heterogeneous target.	Early-phase clinical trials have shown safety but limited efficacy due to antigen loss and tumor heterogeneity.
IL13Rα2	A receptor subunit is overexpressed in recurrent GBM but not in normal brain tissue. It is associated with tumor aggressiveness and poor outcomes, providing tumor specificity for therapies.	CAR-T trials targeting IL13Rα2 have shown dramatic individual responses, including complete remission in one case, though durability remains a challenge.
HER2	A member of the epidermal growth factor receptor family found in ~40% of GBMs. It sustains tumor growth and cell survival and is also targeted in breast cancer, providing translational therapeutic insights.	HER2 CAR-Ts are in early clinical testing; responses are modest but demonstrate feasibility and safety.
GD2	A disialoganglioside is highly expressed in pediatric brain tumors such as diffuse midline gliomas and DIPG. It plays a role in tumor cell adhesion and signaling.	CAR-Ts targeting GD2 have shown promising activity in pediatric gliomas, with ongoing studies exploring applications in GBM.
EphA2	A receptor tyrosine kinase involved in cell migration and invasion. Its overexpression in GBM correlates with tumor invasiveness and poor prognosis.	Preclinical studies support EphA2 as a target; CAR-T trials are underway but still at an early stage.

The table illustrates the wide variety of tumor-associated antigens that have been identified for use in CAR-T cell therapy for patients with glioblastoma multiforme. This also demonstrates how the diversity of the tumor antigens among

individual tumors results in variable responses to CAR-T cell therapy, as no single antigen is used across all the different glioblastoma cells. Antigen heterogeneity helps explain why there is a consistent barrier in achieving a long-term solution, as demonstrated in the early stages of the clinical trials. Due to the antigen heterogeneity, future CAR-T cell strategies will likely require a multi-antigen targeting approach to improve the long-term efficacy and minimize antigen escape.

Mechanism of Action: CAR-T cells target tumor antigens via CARs, which engage cytotoxic activity such as cytokine release and direct tumor killing. GBM is tough, though, as summarized in Table 4.

Table 4: Challenges for CAR-T Therapy in GBM.

Challenge	Description / Impact	Example / Notes
Tumor antigen heterogeneity	GBM cells express different antigens; some may not carry the target CAR-T antigen, leading to incomplete killing.	EGFRvIII-targeted CAR-T may not affect cells lacking EGFRvIII. ¹¹
Immunosuppressive tumor microenvironment	GBM secretes TGF-β, recruits Tregs, and expresses checkpoint proteins, reducing CAR-T cell activation and persistence.	Limits the durability and efficacy of therapy.
Restricted T cell trafficking / BBB	The blood-brain barrier restricts CAR-T cells from efficiently reaching tumor sites when administered systemically.	Strategies like intraventricular or intracranial delivery are being explored.
Risk of cerebral edema and neurotoxicity	Immune activation in the brain can cause swelling or neurotoxic effects, posing safety concerns.	Dose titration and careful monitoring are required in clinical trials.

The table describes the major constraints imposed by using CAR-T Cells to treat solid tumors such as glioblastoma multiforme. It stresses that if we wish to improve the persistence, safety, and efficacy of CAR-T Cell therapy, these constraints must be overcome.

Clinical Trials:

The Major Car-T cell clinical trials discussed in this section will be summarized in Table 5.

GD2 CAR T Cells in Diffuse Intrinsic Pontine Gliomas (DIPG) & Spinal Diffuse Midline Glioma (DMG):

This is a Phase I study testing CAR-T cells targeting GD2 antigen in eleven kids and young adults with a tough type of brain tumor called diffuse midline glioma (including DIPG). They gave the treatment IV first, and if it worked, some patients got more doses directly into the brain. Some patients had brain swelling and inflammation, but the doctors were able to manage it with ICU care. Even with the risks, 4 patients had major tumor shrinkage (one over 30 months), and 9 patients had symptom improvement, so this therapy works very well but needs a phase II.⁴²

EphA2-CAR T Cells in Recurrent Glioblastoma:

Three patients with recurrent glioblastoma were treated with a form of CAR-T cells that were designed to target a protein called EphA2. Each patient received a single dose of these specially made T-cells via IV injection, and each received chemotherapy before the CAR-T cells to condition their bodies. Two of the patients developed cytokine release syndrome (CRS), which caused them to experience breathing problems due to inflammation in the lungs. However, the CRS was con-

trolled with steroid medication. The CAR-T cells were present in the body for several weeks, but the tumors continued to grow in all three cases. In one case, the patient's tumor was stable for a short time; however, the median overall survival among the three cases ranged between 3 and 6 months. The study demonstrated that the treatment is possible and has a level of safety, but it does not currently work very well.⁴³

IL-13Rα2-Targeted CAR-T Cells in Recurrent Glioblastoma:

Doctors conducted a clinical trial involving a total of 65 individuals with recurrent high-grade gliomas. Those in the clinical trial were treated with CAR-T cell therapy that targeted the IL-13Rα2 antigen. In order to treat the patients, the CAR-T cells were administered directly into the tumor, into the spinal fluid surrounding the brain, or both. Overall, the treatment was safe; however, the two most serious adverse reactions experienced during the trial were grade 3 encephalopathy and ataxia. Fifty percent of the patients (29 of 58) were stable or better after receiving the treatment. Within the group of patients that were better off after receiving the treatment, there were two patients who experienced a partial response and another two patients who experienced a complete response (one of the two complete responses was from a patient who received an additional CAR-T after the trial protocol). In total, the median for overall survival among the patients that received the CAR-T cells was 7.7 months; however, the median for overall survival among those patients that received the personalized dual delivery treatment was 10.2 months. The administration of the CAR-T cells resulted in an increase in the levels of pro-inflammatory cytokines within the central nervous system, including IFNγ, CXCL9, and CXCL10. This increase in cytokines indicates that the CAR-T cells were active. Additionally, patients who had tumors that contained more CD3+ T cells than other patients tended to live longer.⁴⁴

HER2-CAR T Cells in Progressive Glioblastoma:

A Phase I clinical trial examined HER2-specific CAR-modified virus-specific T cells (VSTs) in 17 patients with progressive HER2-positive glioblastoma. The VSTs used in this clinical trial were produced from each patient and genetically modified to produce a second-generation HER2-CAR containing a CD28.ζ signaling domain. The patients in the clinical trial received the HER2-CAR VSTs without first undergoing lymphodepletion. The treatment was tolerable in that none of the patients experienced a dose-limiting toxicity. The one patient that achieved a partial response lasted for more than 9 months, while seven of the sixteen patients that were evaluated were able to maintain stable disease for periods of time ranging from 8 to 29 months. Three of the patients in the clinical trial were able to remain free of further disease for periods of time up to 29 months, and the median time until death from the date of infusion was 11.1 months, and the median time until death from the date of diagnosis was 24.5 months. The findings from this clinical trial indicate that HER2-CAR VST therapy is both safe and capable of producing clinical benefits for some patients with glioblastoma, the degree of benefit was relatively

small in many cases, indicating a need for additional strategies to improve the effectiveness of HER2-CAR VST therapy against glioblastoma.⁹

EGFRvIII-CAR T Cells in Recurrent Glioblastoma:

Similar to the above-mentioned clinical trials, a Phase I clinical trial also examined a third-generation EGFRvIII-targeted CAR T product in 18 patients with recurrent glioblastoma. Following a course of lymphodepletive chemotherapy, the patients in the clinical trial received CAR T cells at various doses ranging from approximately 6.3×10^7 to 2.6×10^8 cells along with IL-2 support. The treatment was generally safe; however, serious adverse reactions were noted, including severe hypoxia and treatment-related death in one of the patients who received the higher doses of CAR T cells. There were limited efficacy outcomes noted among the patients in the clinical trial, as evidenced by the fact that the median time until disease progression was only 1.3 months (IQR = 1.1 – 1.9 months) and the median time until death from the date of infusion was 6.9 months. While no objective tumor responses were noted in the majority of the patients in the clinical trial, a couple of long-term outliers survived for more than 1 year, with one patient remaining alive at 59 months. These findings highlight both the biological potential of EGFRvIII-directed CAR T cells, as well as the significant challenges presented by the heterogeneity of glioblastoma, antigen escape, and the immunosuppressive nature of the tumor microenvironment.¹¹

Role of CRISPR in CAR-T:

CRISPR enhances CAR-T therapy through the removal of inhibitory receptors, upregulation of CAR, and enabling future *in vivo* production of CAR-T.^{12,38} Such advances can potentially overcome GBM's immunosuppressive tumor microenvironment and increase the specificity and durability of CAR-T cells.

Table 5: Clinical Trial Highlights.

Trial Name / Identifier	Number of Participants and Patients Population	Treatment Protocol / Delivery Method	Primary Outcomes / Results	Key Notes / Side Effects
GD2 CAR T Cells in DIPG & DMG ⁴² Phase I Trial	11 patients, who included children and young adults with midline glioma	IV infusion first; some received repeat intracerebral doses	4 had major tumor shrinkage (one >30 months); 9 had symptom improvement	Brain swelling, inflammation; higher IV doses caused more toxicity, but were manageable in the ICU
EphA2-CAR T Cells in Recurrent GBM ⁴² Phase I Trial	3 adult patients with recurrent GBM post-standard therapy	Single IV infusion after chemotherapy conditioning	Limited efficacy: tumors mostly progressed; OS 3–6 months; brief stable disease in 1 patient	Cytokine release syndrome in 2 patients (managed with steroids); lung shortness of breath
IL-13Rα2-CAR T Cells in Recurrent GBM ⁴⁴ Phase I Trial	65 patients with recurrent high-grade glioma	Intratumoral, intracerebrospinal, or dual delivery	29/58 patients had stable or improved disease; 2 partial + 2 complete responses; OS median 7.7 months (10.2 in dual-delivery group)	Grade 3 encephalopathy & ataxia in 2 cases; CAR-T activity correlated with cytokine elevation & CD3 T-cell presence
HER2-CAR T Cells in Progressive GBM ⁹ Phase I Trial	17 patients with HER2-positive progressive GBM	Autologous virus-specific T cells engineered with HER2-CAR; IV infusion without lymphodepletion	1 partial response >9 months; 7 stable disease (8–29 months); median OS 11.1 months from infusion, 24.5 from diagnosis	Well tolerated; no dose-limiting toxicities; modest responses suggest need for improved strategies

EGFRvIII (3rd gen)-CAR T Cells in Recurrent GBM¹¹ Phase I Trial	18 patients with recurrent EGFRvIII+ GBM	IV infusion after lymphodepletion + IL-2, doses from 6.3×10^7 to 2.6×10^8	Median PFS: 1.3 months; median OS: 6.9 months; no objective responses; 2 patients > 1 year survival, 1 at 59 months	Generally safe, but serious toxicities include severe hypoxia & 1 treatment-related death at higher doses
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The table outlines the main conclusions drawn from several large-scale studies of CAR-T Cell therapy for glioblastoma multiforme. Although the treatments were found to be relatively safe and biologically effective, they provided little or no significant clinical benefit. Hence, the studies emphasize the necessity for further modification of CAR-T Cell therapy through either the use of combination therapies or genetic manipulation like CRISPR to enhance their therapeutic potential.

■ Discussion

Despite advances in surgical and systemic treatments (radiation and chemotherapy with temozolomide), patients with glioblastoma (GBM) continue to experience short-term survival gains from standard care, and recurrence is almost universal. The failure of current treatments has led to investigations into targeted therapies, specifically CAR-T cell therapy as a form of precision immunotherapy.

While clinical trials using CAR-T therapy for GBM have demonstrated safety and biological activity, it has not achieved long-term therapeutic benefit due to: 1) the heterogeneous nature of tumors; 2) the inability of CAR-T cells to traffic effectively through the blood-brain-barrier; and 3) the suppressive effects of the tumor's immune environment on the function of CAR-T cells. Despite this, many studies have used antigen-specific targeting (EGFRvIII, IL13R α 2, HER2, EphA2, GD2), resulting in only transient anti-tumor responses and localized tumor regression or symptom relief. Antigen escape and heterogeneous expression remain significant barriers to successful CAR-T therapy.

CRISPR-Cas9 gene editing technology has the potential to overcome the limitations associated with CAR-T therapy. In preclinical trials, CRISPR has been able to increase CAR-T persistence, eliminate inhibitory checkpoint molecules (PD-1), and enable more consistent CAR expression.^{9,38} New methods of delivering CRISPR constructs across the blood-brain barrier using nanocarriers and exosomes have also become available. Although many applications of CRISPR technology are currently experimental, it is expected to improve both the efficacy and safety of CAR-T therapies for GBM.

The intersection of CAR-T cell therapy and CRISPR-based engineering presents a new and exciting area of research. Future clinical evaluations will need to evaluate combinations of CAR-T therapies (i.e., multi-antigen targeting and/or checkpoint inhibition) to overcome the complex immunological environment of GBM.

The following are several limitations of this review. First, few CAR-T clinical trials have been published in GBM with heterogeneous small sample sizes, study design, antigen target, and route of administration, making comparison challenging. Second, the majority of CRISPR studies are preclinical and thus results will not likely be extrapolated directly to patients

due to heterogeneity in tumor biology and the blood-brain barrier. Third, publication bias would also be a risk because positive findings are more likely to be published than null or negative findings. Finally, while every effort was made to obtain all relevant studies using systematic searching, there is the potential that some new studies or non-English studies were not captured.

■ Conclusion

Glioblastomas (GBMs) are cancers that have an extremely poor prognosis and cannot currently be cured by any established standard treatments. CAR-T cells have shown safety and early signs of efficacy in treating glioblastoma; however, CAR-T has not yet demonstrated its ability to produce long-lasting or durable responses against this cancer type. The gene editing capability of CRISPR could potentially enhance the ability of CAR-T cells to target glioblastoma tumors by addressing the major hurdles associated with tumor heterogeneity, immune suppressive mechanisms, and persistence of CAR-T cells. As additional clinical trials are completed, the application of CRISPR-based modifications to CAR-T cells may usher in a new era of glioblastoma treatment. It will be necessary to employ multi-modality approaches using genetic engineering, immunomodulation, and targeted therapy to provide significant improvement in the overall survival rates of patients diagnosed with glioblastomas.

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Development of a Novel Polymer-based Drug Delivery System

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ABSTRACT: Oligonucleotide drugs hold transformative potential in therapeutics by addressing a range of diseases with unmet medical needs. However, clinically, they are hindered by limitations in membrane permeability and biodistribution, underscoring the need for optimized delivery platforms. In this study, we developed a novel bottlebrush polymer-based delivery system featuring cholesterol molecules on its side chains to facilitate cellular uptake and broaden biodistribution. The design involved the conjugation of a single cholesterol molecule per bottlebrush polymer, followed by PEGylation to increase solubility and biocompatibility. Gel permeation chromatography (GPC) confirmed a consistent molecular weight of approximately 300 kDa of the sample. At the same time, Transmission Electron Microscopy (TEM) revealed a uniform spherical nanostructure with a core diameter of around 20 nm. Cellular uptake assays demonstrated significantly improved uptake of Cy3-labeled bottlebrush polymers compared to unmodified controls, validating the efficacy of cholesterol incorporation. These findings suggest that the cholesterol-modified bottlebrush polymer platform represents a promising and adaptable delivery system for oligonucleotide-based therapies.

KEYWORDS: Biomedical Engineering, Biomaterials and Regenerative Medicine, Bottlebrush Polymer, Oligonucleotide, Cellular Uptake.

■ Introduction

Oligonucleotide drugs hold significant promise for revolutionizing disease therapeutics due to their ability to target a broad spectrum of diseases with high molecular specificity.¹ These therapies have enabled breakthroughs in previously intractable conditions, exemplified by approved drugs such as Spinraza for spinal muscular atrophy and investigational treatments for myotonic dystrophy type 1 (DM1). Such advancements highlight the potential of oligonucleotide-based therapies for precise modulation of genes and molecular pathways.² However, the clinical translation of oligonucleotide drugs remains limited by inherent structural and physicochemical properties, such as a polyanionic backbone that confers a strong negative charge.³ This feature severely restricts membrane permeability and cellular uptake, posing substantial challenges to effective systemic delivery. As a result, the development of optimized and versatile delivery platforms remains a critical focus in advancing oligonucleotide-based therapeutics.

To address these challenges, recent studies have explored various nanoparticle-based delivery systems. Lipid nanoparticles (LNPs), for instance, have shown promise by encapsulating oligonucleotides within a lipid shell, enhancing their stability and facilitating uptake into target cells.⁴ However, LNPs tend to accumulate in the liver and spleen due to their size and composition, which can restrict therapeutic reach to non-hepatic tissues and limit their utility in targeting diverse organ systems.⁵ To address this problem, oligonucleotides can be conjugated to targeting moieties such as small molecules, peptides, and antibodies, which can greatly enhance target specificity and mitigate off-target effects.⁶ Antibody-conjugated delivery systems represent further refinement, leveraging the specificity

of antibodies to target oligonucleotides to particular cell types or tissues. While this approach improves targeted delivery, it is in some cases hampered by limited blood circulation times—often due to linker instability and Fc-mediated clearance—as well as challenges in maintaining oligonucleotide stability. This results in decreased efficacy in delivering therapeutics to certain tissues or in conditions that require prolonged systemic exposure.⁷

To address these limitations, a bottlebrush polymer delivery system was developed to improve cellular uptake of oligonucleotide drugs and prolong blood circulation time.⁸ Unlike traditional oligonucleotide delivery systems that predominantly target the liver, this platform demonstrates potential for broader organ targeting, expanding its therapeutic applications.^{9,10} To further enhance cellular uptake and biodistribution, a new generation of bottlebrush polymer was designed and synthesized. This updated platform was thoroughly characterized to confirm its structural properties and optimize its suitability for targeted delivery.

■ Methods

Oligonucleotides and poly(serinol phosphodiester) monomer synthesis:

The synthesis (Figure 1) of serinol phosphoramidite started from β -alanine-serinol (compound 1), where 1 mmol of compound 1 was dissolved in 30 mL of pyridine. 4,4'-Dimethoxytrityl chloride was added dropwise under an ice bath. The reaction was carried out overnight and was quenched with methanol. The resulting mixture was purified by column chromatography using an ethyl acetate: hexane solvent system (2:1

volume ratio) to give rise to serinol-DMT (compound 2) as a white crystal with a 63% yield.

The synthesized compound 2 (1 mmol) was subsequently dissolved in dichloromethane at room temperature, followed by the addition of *N,N*-diisopropylethylamine (5 mmol). 2-cyanoethyl-*N,N*-diisopropylchlorophosphoramidite (2 mmol) was added dropwise under an ice bath. The reaction was stirred for 1 hour and quenched with 1 mL of ethyl acetate. The resulting mixture was purified by column chromatography (gradient elution using ethyl acetate: hexane from 1:2 to 2:1 v/v) to afford serinol phosphoramidite (compound 3) as a white crystal with a 75% yield.

Using a similar procedure, Compound 5 was synthesized from Compound 4 with a yield of 65%.

Oligonucleotides and poly(serinol phosphodiester) backbone synthesis:

All oligonucleotides, with or without poly(serinol phosphodiester) backbones, were synthesized using solid-phase synthesis on a Model 391 DNA synthesizer (Applied Biosystems, Inc., Foster City, CA). Both natural and modified DNA phosphoramidites were sourced from Glen Research Co. (Sterling, VA, USA). Following synthesis, these oligonucleotides underwent on-column deprotection with 20% piperidine in DMF three times to remove the phosphate protection groups, followed by two washes with DMF.¹¹ Each oligonucleotide strand was cleaved from the CPG column and deprotected in an aqueous ammonium hydroxide solution (28-30% NH₃) at room temperature for 24 hours. The sequence of the DNA oligonucleotide is *CAG AGG AGT ATT AGG ACT*.

Purification in the Glen-Pak™ DNA purification cartridge:

All oligonucleotide strands were purified using Glen-Pak™ DNA purification cartridges. Following purification, the purified oligonucleotide solution was lyophilized in preparation for the subsequent PEGylation reaction.

PEGylation of pacDNA:¹¹

To conjugate the PEG side chains, 100 nmol of the oligonucleotides with poly(serinol phosphodiester) modified backbones and 30 mg of N-hydroxysuccinimide (NHS)-terminated 10 kDa mPEG were dissolved together in 1 mL of phosphate-buffered saline (PBS, pH 7.4). The solution was gently shaken at 4°C overnight and then lyophilized into a white powder, which was dissolved in 1 mL of anhydrous DMF as well as 42 µL triethylamine. Subsequently, 30 mg of NHS-PEG in 1 mL DMF was added in 5 aliquots at 12-hour intervals. After the last addition, the reaction mixture was shaken at 25 °C for an additional 12 h, followed by lyophilization.

Purification in GPC:¹²

After complete PEGylation, the molecular weight of pacDNA can reach approximately 250 kDa, allowing it to be separated from free 10 kDa PEG and unreacted free oligonucleotides via fractionation during aqueous gel permeation chromatography (GPC).

Transmission electron microscope:¹³

Following purification by GPC, transmission electron microscopy (TEM) was performed, which provided visual evidence of uniform, spherical nanostructures with an average core diameter of 20 nm, confirming the controlled assembly achieved through the synthetic process.

Cellular uptake assay:¹⁴

Cellular uptake of the samples and controls was evaluated via flow cytometry. Cells were plated in 24-well plates at a density of 2×10^5 cells per well in 1 mL of complete growth medium and incubated for 24 hours at 37 °C with 5% CO₂. After washing with 1×PBS, conjugate samples and controls (concentrations ranging from 250 nM to 5 µM DNA equivalents) were prepared in 400 µL serum-free culture medium and added to each well. Cells were then incubated at 37°C for an additional 4 to 6 hours. Following incubation, cells were washed twice with PBS and treated with 60 µL of trypsin per well. Afterward, 1 mL of PBS was added to each well to resuspend the cells. Finally, cells were analyzed using an Attune™ NxT flow cytometer (Invitrogen, MA), with data collected from 10,000 gated events per sample.

Results and Discussion

Results:

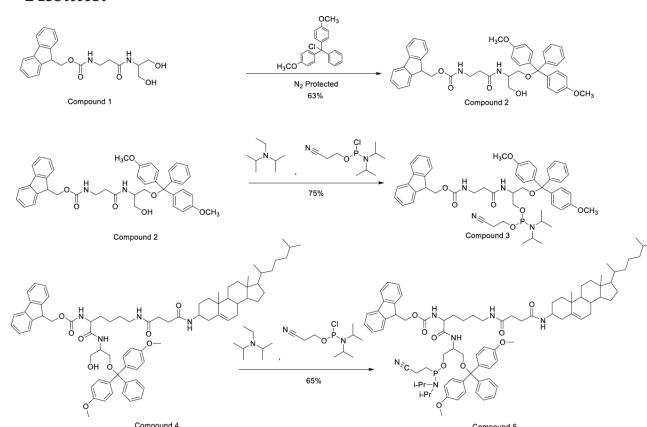


Figure 1: Synthesis of serinol and cholesterol phosphoramidite monomers enabling bottlebrush polymer functionalization. These monomers provide the chemical basis for controlled incorporation of cholesterol into the polymer backbone.

In collaboration with lab colleagues, serinol phosphoramidite (compound 3) was synthesized in a two-step organic synthesis from a protected diol (compound 1), while cholesterol phosphoramidite (compound 5) was prepared via a one-step phosphitylation reaction. The synthetic routes are shown in Figure 1. These monomers were subsequently used in solid-phase synthesis to generate bottlebrush polymer-oligonucleotide conjugates with defined cholesterol content. Successful synthesis enables precise structural modification of the polymer platform for downstream characterization and biological evaluation.

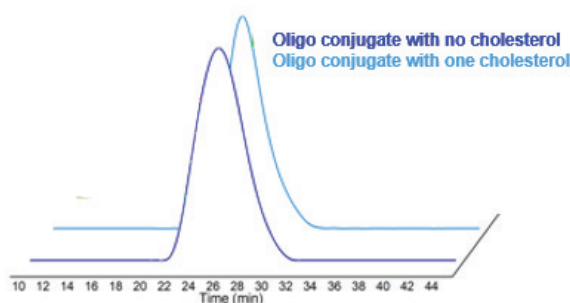


Figure 2: GPC analysis confirming successful cholesterol conjugation and purification of bottlebrush oligonucleotide conjugates. The chromatographic profiles indicate formation of uniform polymer populations following conjugation.

Following conjugation, the cholesterol-modified conjugate was purified by GPC. Two oligo conjugates were generated: one without cholesterol and one containing a single cholesterol unit, as shown in Figure 2. Elution profiles are plotted as detector response versus retention time. Both conjugates exhibit narrow, single peaks, indicating uniform molecular weight distributions. The retention shift observed for the cholesterol-modified construct confirms successful incorporation of the hydrophobic moiety without evidence of aggregation or degradation.

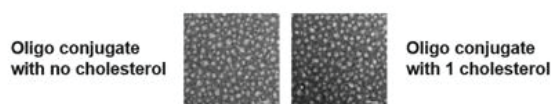


Figure 3: TEM of selected oligo cholesterol conjugates demonstrating uniform nanoparticle morphology of cholesterol-modified bottlebrush conjugates. The observed structures confirm consistent nanoscale assembly after cholesterol incorporation.

Oligonucleotide conjugates with and without cholesterol modification were imaged by TEM for particle size and morphology analysis, as shown in Figure 3. Images represent purified samples following GPC. Both constructs form uniform spherical nanostructures with an average core diameter of approximately 20 nm. Particle size and morphology were consistent across samples. These results demonstrate that cholesterol incorporation preserves nanoparticle structure and does not disrupt controlled assembly of the bottlebrush platform.

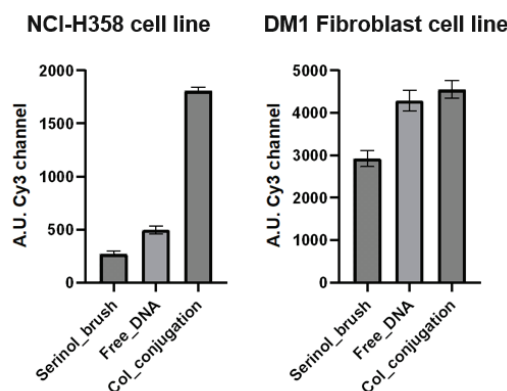


Figure 4: Enhanced cellular uptake of cholesterol-conjugated bottlebrush polymers in NCI-H358 and DM1 fibroblast cells. Cholesterol modification improves intracellular delivery compared to unmodified controls.

The cholesterol-conjugated oligonucleotide was evaluated in NCI-H358 and DM1 fibroblast cells using an unloaded serinol brush and free DNA as controls, as shown in Figure 4. Cellular uptake was quantified by flow cytometry as Cy3 fluorescence intensity following 4–6 hours of incubation. Flow cytometry analysis revealed significantly increased fluorescence intensity for cholesterol-modified constructs compared to controls in both cell lines. These results confirm that cholesterol conjugation substantially enhances cellular uptake and improves the delivery efficiency of the bottlebrush polymer system.

Discussion:

In this study, we successfully synthesized a bottlebrush polymer monomer featuring cholesterol modification on its side chain, followed by the conjugation of one cholesterol unit per polymer. Subsequent PEGylation was completed, and gel permeation chromatography (GPC) analysis confirmed that all resulting polymers exhibited a molecular weight of approximately 300 kDa, indicating successful conjugation and size consistency across samples.

Transmission Electron Microscopy (TEM) provided visual confirmation of the bottlebrush polymers' uniform, spherical nanostructures with an average core diameter of 20nm, reflecting the controlled assembly achieved through our synthetic approach. This spherical morphology, combined with cholesterol conjugation, likely plays a key role in enhancing cellular uptake, as cholesterol's affinity for cellular membranes facilitates closer interaction and internalization.¹⁵

Flow cytometry-based cellular uptake assays further supported the efficacy of this design. Cy3-labeled bottlebrush polymers demonstrated notably higher cellular uptake compared to controls, demonstrating that the synergistic effect of PEGylation and cholesterol modification improves delivery efficiency. This study focused on constructs containing a single cholesterol unit per polymer; future work could explore multiple cholesterol incorporations to evaluate potential effects on uptake and biodistribution. Overall, this study underscores the potential of cholesterol-modified bottlebrush polymers as effective carriers for oligonucleotide therapeutics, offering improved membrane permeability, favorable biodistribution, and opportunities for targeted, non-hepatic delivery.

Conclusion

A cholesterol-modified bottlebrush polymer platform was developed and characterized for the enhanced delivery of oligonucleotide drugs. The synthesis process involved conjugating one cholesterol unit per bottlebrush polymer monomer, followed by PEGylation, yielding polymers with consistent molecular weights of approximately 300 kDa, as confirmed by GPC. TEM analysis revealed uniform, spherical nanostructures, indicating stable and controlled assembly. Cellular uptake assays further demonstrated significantly enhanced uptake, likely due to the membrane affinity imparted by cholesterol conjugation.¹⁵ This innovative bottlebrush polymer delivery system addresses the challenges related to membrane permeability and has the potential to improve biodistribution,

offering a promising approach for targeted oligonucleotide delivery beyond hepatic tissues and expanding the potential for therapeutic applications.

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Bacteriophage Therapy: A Double-Edged Sword in Modern Medicine

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ABSTRACT: Invented in the 20th century, antibiotics revolutionized the treatment of bacterial infections and remain the standard of care today. However, the rising threat of antimicrobial resistance (AMR) has renewed scientific interest in phage therapies (PTs). PTs are treatments that employ bacteriophages, viruses that naturally target bacteria, and could be used as alternatives to antibiotics. While PTs were developed around the same time as antibiotics, they fell out of use in Western medicine due to logistical and regulatory factors. Recent research underlines advantages that PTs have been purported to have, including self-replication capabilities, high specificity, biofilm penetration, and synergistic activity when administered alongside antibiotics. Nonetheless, limitations remain, such as complex formulation requirements, pharmaceutical unknowns, and significant regulatory hurdles. Clinical trials indicate that while PTs are generally safe and effective, they are not reliably superior to antibiotics. Additionally, methodological inconsistencies between studies hinder data synthesis, indicating that the field is still in its infancy. Surveys on current patient and clinician attitudes indicate that while many are receptive to trying PT, most are not initially familiar with PT. Overall, while PT is a promising response to AMR, trial standardization, consistent clinical protocols, and regulatory development are still necessary for it to achieve more prolific use.

KEYWORDS: Biomedical and Health Sciences, Genetics and Molecular Biology of Disease, Bacteriophage Therapy, Antimicrobial Resistance.

■ Introduction

Following their discovery in the 20th century, antibiotics rapidly became the standard treatment for bacterial infections. However, bacteria soon began to develop resistance to these new drugs. Antibiotic resistance has rapidly emerged as one of the most pressing concerns of the 21st century. The widespread and often indiscriminate use of antibiotics in clinical settings has accelerated the evolution of antibiotic-resistant strains of pathogenic bacteria.¹ As a result of this, previously treatable infections are becoming increasingly difficult to tackle. Currently, 700,000 annual deaths are a result of antibiotic-resistant bacteria (ARB), and this figure is projected to increase to over 10 million deaths per year by 2050.²

As a response to the diminishing effectiveness of traditional antibiotics, new research is exploring bacteriophage therapies as a mechanism to combat ARBs or work as an adjunct treatment with antibiotics to produce enhanced synergistic results.³ Bacteriophages—viruses that specifically work to infect bacteria—were first discovered over a century ago, and were originally used therapeutically before antibiotics became widespread.⁴⁻⁶ Now, with the growing ARB crisis, phages are being revisited as a mechanism to target specific pathogens, including ARBs, without disturbing the broader microbiome.^{5,7}

Despite their advantages, phage therapies (PTs) also have their drawbacks. One concern stems from the potential that bacteria could evolve to be resistant to phages, similarly to how they are currently resistant to antibiotics.⁸ Additionally, although current results seem promising, the safety of phage therapy in human clinical applications still appears to be some-

what uncertain, as noted by reviews conducted on animal and human *in vivo* studies.⁹ Finally, there is also the risk that phages may indirectly facilitate the spread of bacterial antibiotic resistance genes through horizontal gene transfer, more specifically through transduction—a process in which phages can package and transfer genetic material, including antibiotic resistance genes (ARGs), and transfer them between bacteria, which can rapidly accelerate the spread and dissemination of ARBs.¹⁰⁻¹²

Based on this, it is apparent that while phages may be seen by some as a viable alternative to antibiotics in clinical settings, it is also true that their exact efficacy is somewhat uncertain. As a result of this dilemma, bacteriophage therapies currently stand as a promising but uncertain solution for combating antimicrobial resistance (AMR).¹⁰

This literature review aims to discuss the mechanisms behind phage therapy; explore the advantages and disadvantages of phage therapy to combat antibiotic resistance; examine studies of human clinical applications of phage therapy compiled across various databases; discuss current attitudes towards phage therapy; and discuss avenues for future research and clinical implementation of phage therapy to combat AMR.

■ Methodology

The objective of this study was to synthesize existing evidence regarding the evolution of PTs, their advantages, their disadvantages, current clinical trial results, and current clinical attitudes towards PT. This study was a literature review that employed a comprehensive search of databases—PubMed, ScienceDirect, and Clinicaltrials.gov—to identify and select papers

for analysis. For PubMed, search terms such as *phage therapy* and *antibiotic resistance* were used to narrow down results, as well as filters that restricted the selection of papers to only those covering human clinical applications, such as clinical trials and case reports. For ScienceDirect, similar search terms were used, and filters were applied to restrict the selection to only research articles. Finally, Clinicaltrials.gov was searched using filters restricting the “Intervention/treatment” to only “Phage Therapy.” All three databases were also filtered to ensure that only English-language articles published after 2020 would appear.

The papers from all three databases were then compiled in a spreadsheet in order to refine the selection. Duplicates were removed, as were papers that did not have the full body of text available, did not include their results, or did not directly concern phage therapy, antibiotic resistance, or ARGs. Veterinary trials were also excluded, as were studies aiming to characterize the bacteriophage itself as opposed to its applications in human clinical therapy.

Following this, a final selection of papers was compiled and analyzed. This involved the extraction of information on the historical development of antibiotics and phage therapies, the mechanisms behind both, the mechanisms behind antibiotic resistance, details pertaining to the benefits and limitations of phage therapy, patient and disease characteristics from PT trials, details of various phage treatments, and patient outcomes from PT.

No physical tools or materials were used in this study, as only online sources were consulted. Additionally, because this was a literature review, formal ethical approval was not required.

■ Discussion

The Basics of Bacteriophages:

Bacteriophages, often referred to as “phages,” are a subclass of viruses that proliferate by infecting and replicating in bacteria. They are the most abundant biological entities on Earth, as they are found anywhere bacteria can exist, with the population containing an estimated $\sim 10^{31}$ particles.¹³

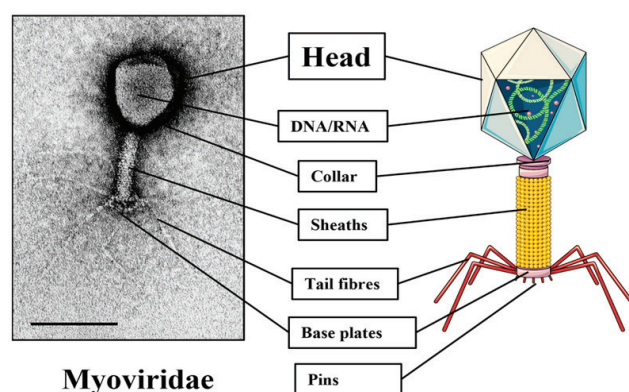


Figure 1: Structure of a Phage. This figure shows a simplified diagram of a bacteriophage, including the head, DNA/RNA, collar, sheaths, tail fibers, base plates, and pins, which collectively allow for genome injection and bacterial cell lysis. These structural features guide the mechanistic basis of bacteriophages' therapeutic action. Adapted from <https://doi.org/10.3390/coatings13030609>.

As seen in Figure 1, structurally, phages are relatively simple; they are composed of genetic material (DNA or RNA, which can be either double or single stranded) that is enclosed in a capsid—an outer protein shell.¹⁵ Phages may also possess elaborate structures such as contractile tails, base plates, pins, and tail fibers, which aid in host recognition and DNA injection. In the specific case of bacteriophages, they have tails that assist the phage in host recognition using receptor-binding proteins, as well as attaching and injecting material into the host. In the case of phage therapies, the host is the pathogenic bacterium being targeted by the therapy. After this step, the phage hijacks its host's cellular machinery to reproduce. Primarily, phages accomplish this in one of two ways, following either the lytic cycle or the lysogenic cycle.

In the lytic cycle, the virus's genetic material enters the cell, and cellular structures are rapidly redirected to new phage components, which are then assembled into viral progeny. This eventually leads to the host cell being either actively or passively lysed, and the progeny phages go on to infect other bacterial cells similarly. In the lysogenic cycle, phage genetic material is instead incorporated into the host's own genetic material or exists as an episomal plasmid. From there, the phage's genetic material is replicated by the bacteria and passed down to each bacterial daughter cell, progressively infiltrating the population of bacteria. These integrated phage genomes are called prophages. Prophages can convert to the lytic cycle, usually triggered by environmental changes.^{15,16}

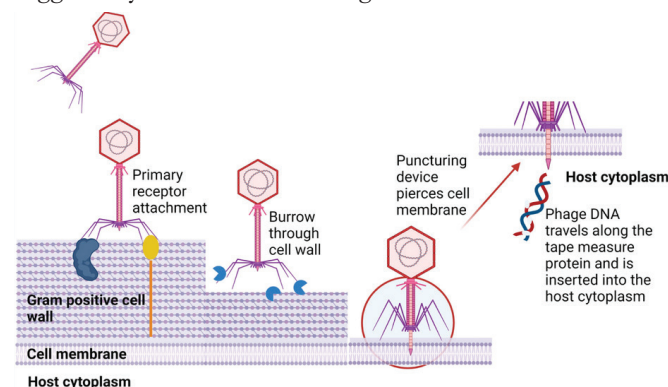


Figure 2: Process of Bacteriophage Infection. The figure demonstrates how bacteriophages rely on primary receptor attachment to infect host cells. The reliance on receptor-mediated attachment is the basis of both the precision of phage targeting and the narrow host range that complicates broader clinical applications. This image is adapted from <https://doi.org/10.3390/v16030478>.

When phages infect bacterial cells, they rely on highly specific receptors to gain entry into the cell. This process is illustrated in Figure 2, which demonstrates how infection of the bacterial cell cannot occur without proper receptor attachment. Due to this, each phage can only target a narrow range of hosts. As a result of this, they are particularly appealing for clinical therapies because of their potential to provide highly targeted action against pathogenic bacteria while leaving the rest of the microbiome otherwise unchanged.⁵ However, they also act as a double-edged sword in the sense that the narrow host range may make it difficult to select phages for therapies.

Development Strategies of Phage Therapies:

Most bacteriophage therapies do not rely on one single phage, but instead use a combination of several phages known as phage cocktails. This is done for two primary biological realities. Firstly, phages have narrow host ranges due to their high receptor specificity, and although this can be an advantage when seeking targeted therapies, it also means that for complex infections, multiple types of bacteriophages will need to be employed to produce an effective therapy. Secondly, bacteria evolve resistance rapidly when subject to selective pressures, meaning that there is always the chance for a course of phage therapy to become ineffective against a bacterial strain that develops resistance. Having multiple phages in the cocktail mitigates the risk of resistance developing, depending on the bacterial context.¹⁸ However, there are still some phage therapies that have utilized singular phages.

There are several different categories of phage therapy. One such category is “off-the-shelf” therapies, or fixed therapies, which are composed of predetermined phages that cover a predicted range of bacteria. The phages are screened for host-range breadth before being formulated into the cocktail. They are usually manufactured and then stocked by pharmaceutical isolation facilities, allowing for immediate use without having to isolate the pathogen.^{19,20} This approach is attractive because it fits neatly into existing guidelines for drug approval pathways.

There are also modifiable phage therapies, which can be reviewed and adapted as needed. These resemble fixed cocktails, but they differ in the sense that they are periodically reformulated in order to retain efficacy against dominant clinical strains or as resistance patterns shift. Phages can be added, replaced, or modified to broaden the host range. This is a strategy that was commonly used in Georgia and Eastern Europe.²¹

Phage therapies can also come as personalized phage preparations. Phages are drawn from phage banks and then assayed against captured and cultured samples of a patient’s infecting bacterial strains to see how well they respond to that particular patient’s bacterial infection before later being synthesized into the cocktail, making for a more targeted treatment. The main consideration with this strategy seems to be the time it will take to prepare the treatment, but host range breadth is not a concern because specificity is the goal.

Genetic engineering, such as CRISPR gene editing or *in vitro* evolution (“phage training”), can also be used to broaden or optimize phage host range before the phages are incorporated into cocktails.²² Engineered phages may also be constructed to express biofilm-degrading enzymes or to help enhance host cell killing ability. These modified phages will then be incorporated into the cocktail.

In practice, modified and fixed therapies will likely offer more efficient and immediate access to the treatment while also being compatible with regulatory guidelines. Personalized and engineered therapies allow for greater adaptability and specificity while also being more time-consuming and labor-intensive to produce.

Advantages of Phage Therapy:

There are many advantages that phage therapies have been purported to have over traditional courses of antibiotics. For instance, the fact that bacteriophages have such high host specificity means that phages can treat infections without wiping out the existing microbiome of a patient. This greatly reduces the risk of secondary infections by bacteria such as *Clostridium difficile*, which can occur after disruptions of the gut microbiome.²³ Additionally, their high host specificity is something that makes them particularly appealing when targeting antimicrobial-resistant bacteria, as genetic engineering can produce phages that are highly attuned to these resistant bacteria.⁵

Furthermore, lytic phages will replicate at the site of infection. This means that phage doses can amplify themselves, providing continuous therapeutic action and reducing the necessity of frequent readministrations. Once the infection has been eliminated, the phages will no longer have any hosts to infect, and consequently, their reproduction will halt, and they will be naturally cleared from the body.

Phages have also been proven to be highly effective against biofilms. Biofilms are described as communities of microbes that stick to a surface. These communities are encased in protective matrices and are often highly resistant to both the immune system and antibiotics. This is of particular importance for devices such as prosthetic implants and catheters, which are breeding grounds for biofilm development. Phage therapies, however, have been shown to penetrate and destroy these biofilms effectively.²⁴

The coevolutionary nature between bacteria and bacteriophages also makes them uniquely suited to address the issue of antibiotic resistance. For instance, if a bacterium were to develop resistance to an antibiotic, an entirely new variety of antibiotic would have to be engineered or introduced in order to treat the infection. This process can take years or even decades and contrasts with bacteriophages, which can be easily genetically engineered to combat the resistant strain.⁸ Phage therapy has also been shown to work synergistically with antibiotics, with combination therapy making treatments more effective and making it difficult for bacteria to develop resistance to either agent alone.³

Overall, these findings suggest that phage therapies offer several advantages over antibiotics in a variety of scenarios. Specifically, their narrow host range, self-replicating capabilities, and synergistic activity when applied alongside antibiotics prove them to be a promising adjunct or alternative treatment in situations where standard therapies may fail.

Disadvantages of Phage Therapy:

As previously mentioned, the high host specificity of bacteriophages can be simultaneously appealing and challenging. The process of finding phages that are uniquely suited to targeting specific bacterial strains can be time-consuming, costly, and especially troublesome in cases involving polymicrobial infections or in acute situations where immediate treatment is a necessity.²⁵ For phage therapy to be effective, hospitals would need to maintain large and well-characterized phage libraries

to maximize the chance of finding suitable matches for any given infection. Such an endeavor is complex and often unrealistic.

Phages also come with many pharmacokinetic unknowns. Firstly, the body's immune response might counteract the therapeutic effects of phages, as it is trained to recognize and clear phages. The body may develop antibodies that work against phages because phages are composed of densely packed genetic material and a protein coat, which can reduce phage efficacy with subsequent treatments.²⁶ There is also the issue of unpredictability, in that, while antibiotics and other drugs can be approached with knowledge of a reliable half-life, phages can replicate at the site of infection, leading to variability in their exact distribution. Additionally, there is still clinical uncertainty concerning how phages interact with deep-seated infections or how they may penetrate certain tissues.²⁷

Bacteriophage therapies also have the potential to cause some adverse effects. For instance, in the process of lysing bacteria—specifically gram-negative bacteria—the phages may release bacterial endotoxins into the system, leading to symptoms such as fever, chills, or septic shock.²⁸⁻³⁰ There is also the theoretical concern that a lysogenic phage could transfer genes that encode for antibiotic resistance between bacteria. This occurs because lysogenic phages, unlike lytic phages, can incorporate genetic material into the bacterial genome before replication and can therefore contribute to bacterial transformation.¹⁵ However, this risk is largely mitigated by the fact that most phage therapies make use of lytic phages.^{18-20, 28-34}

Lastly, there is the issue of regulatory hurdles. There is trouble regarding the purity and standardization of phage therapies, as a phage preparation can be composed of bacterial debris and toxins from the manufacturing process, which could be incorporated into the final product.²⁵ The current regulatory framework for most drugs is also not amenable to phage therapy. Guidelines are often designed around data from a single, standardized product, which proves to be a problem as wide varieties of phage therapies, such as modifiable therapies and personalized therapies, rely on a changing formula.

Despite the therapeutic potential of phage therapies, these limitations indicate that significant scientific, logistical, and regulatory challenges still hinder broader clinical usage. This suggests that further clinical research and evaluation are necessary to determine whether the practical constraints of phage therapy can be outweighed by its benefits.

Currently, modern medicine relies heavily on antimicrobial therapies to support vital procedures, including surgery, chemotherapy, organ transplantation, and intensive care, all of which depend on the reliable mitigation of infectious bacteria.¹ For this reason, despite the uncertainties surrounding phage therapy, as antimicrobial resistance continues to compromise medical procedures, interest in alternative therapies such as phage therapies has increased.¹³

Review of Clinical Studies:

Initially, a total of 368 articles were identified from PubMed, Science Direct, and Clinicaltrials.gov. After analyzing and removing articles that did not meet the inclusion criteria or met

the exclusion criteria, 10 articles were identified as suitable for review. While several studies originated from PubMed or Science Direct, only 1 study was retained from Clinicaltrials.gov, as all others were either incomplete, terminated, had an unknown status, did not publish their results, or had duplicates present in the other databases. Additionally, eight of the ten studies involved clinical trials,^{19, 20, 28-33} while two were case reports examining the treatment of a single patient.^{18, 34}

Their results have been compiled as seen below. “PFU” indicates plaque-forming units, a measurement of the quantity of infectious particles. In this case, it indicates the number of phages given to each patient during phage treatment.

Table 1: Patient Demographics and Details of Infection in Studies Involving Phage Therapy. This table summarizes the pathogens, infection types, sample sizes, and patient histories involved in each of the reviewed studies.

Authors	Pathogen(s)	Infection Type	Sample Size	Key Patient Characteristics
Li et al. ¹⁸	MDR <i>K. pneumoniae</i>	Pulmonary	1	Inpatient, multi-drug resistant infection
Leitner et al. ¹⁹	<i>Enterococcus</i> , <i>E. coli</i> , <i>P. aeruginosa</i> , <i>Staphylococcus</i> , <i>Streptococcus</i>	UTI	97	Men undergoing TURP
Sarker et al. ²⁰	<i>E. coli</i> (ETEC, others)	Acute diarrhea	120	Hospitalized children
Fedorev et al. ²⁸	<i>Staphylococcus</i> spp.	Hip PJI	55	Post-hip replacement infection
Kim et al. ²⁹	<i>E. coli</i>	Uncomplicated UTI	39	Adult females; prior drug-resistant UTI
Jault et al. ³⁰	<i>P. aeruginosa</i>	Burn wound	27	Adults with infected burns
Chan et al. ³¹	<i>P. aeruginosa</i>	Cystic fibrosis (CF) sputum infection	8	CF patients
Gupta et al. ³²	Mixed bacteria	Chronic wounds	20	Nonhealing wounds not responding to conventional debridement
Wright et al. ³³	<i>P. aeruginosa</i>	Chronic otitis	24	Refractory otitis externa
Nick et al. ³⁴	<i>M. abscessus</i>	Pulmonary (CF)	1	Severe cystic fibrosis with multidrug-resistant <i>P. aeruginosa</i>

The demographic data from Table 1 indicate that the majority of recent phage therapy trials involve adults, although there were a few studies that involved children with chronic diarrhea or children with chronic otitis.^{20, 33} Looking at the patients' medical histories, it is clear that phage therapy was often used as a last resort drug to treat drug-resistant and recurrent infections, or in cases where standard therapies had proven ineffective.^{18, 19, 29, 32, 34} Instances of trials involving chronic conditions—such as cystic fibrosis, joint replacement, chronic wounds, or chronic otitis—are also prevalent, indicating phage therapy is being tested in settings where biofilms or resistance would complicate standard care.^{28, 31-34}

Table 2: Outcomes of Phage Treatment in Studies Involving Phage Therapy. This table compares treatment outcomes and safety profiles across the reviewed studies.

Authors	Adverse Events	Treatment Outcome
Li et al. ¹⁸	None reported	Clinical improvement; emergence of phage-resistant strains with single phage treatment, but treatment with the phage cocktail resulted in improvement
Leitner et al. ¹⁹	No serious AEs	Non-inferior to antibiotics; not superior to placebo
Sarker et al. ²⁰	None attributable	No clinical benefit of either phage treatment or standard of care
Fedorev et al. ²⁸	2 instances of transient fever	Phage therapy displayed a lower relapse rate compared to antibiotics
Kim et al. ²⁹	Mild AEs; more with higher dose	Rapid bacterial clearance; symptom resolution
Jault et al. ³⁰	Similar AEs to the standard of care	Slower bacterial reduction than the standard of care
Chan et al. ³¹	2 pulmonary exacerbations, 1 hospitalization	No superiority compared to placebo
Gupta et al. ³²	None reported	Significant wound healing
Wright et al. ³³	None related	Reduced bacterial counts; symptom improvement
Nick et al. ³⁴	Neutralizing antibodies to 1 phage (did not prevent benefit)	Sustained clinical improvement

Table 2 indicates that overall, adverse events were uncommon and generally mild. Some mild AEs include tachycardia and febrile chills,²⁹ pulmonary exacerbations,³¹ transient fever,²⁸ and other events.³⁰ Multiple studies indicated that no adverse events occurred.^{18,20,32,33} Nick *et al.* reported neutralizing antibodies but without any loss of benefit.³⁴

There were several trials that produced favorable results with phage therapy.^{18,28,29,32-34} These are indicated by reduced bacterial counts, minimal relapse rates, and clinical improvement as indicated in the table.

Other trials indicate that phage therapy does not have a significantly higher efficacy than standard care, antibiotics, or placebo treatments. These include Chan *et al.*, where both placebo and study groups performed similarly, but the placebo groups reported a higher quality of life.³¹ Leitner *et al.* and Sarker *et al.* both report no significant advantages or disadvantages to using phage therapy over other treatments.^{19,20} Jault *et al.* notes that while phage therapy had a similar success rate to the standard of care, the phage therapy group had a much slower median reduction rate of bacterial burden, and that low concentrations had insufficient efficacy.³⁰ Kim *et al.* observed a similar linkage between dosage and treatment efficacy, noting that patients experienced more adverse events at higher doses of phage treatment.²⁹

Sarker *et al.* was the only study in which phage therapy produced no notable improvement in the patient at all.²⁰ However, this result was attributed to the fact that only half the planned patients were enrolled by the interim analysis, and previously, they had observed no *in vivo* replication of the phage, which would significantly inhibit the therapy's effectiveness. On the other hand, most other studies performed or referred to *in vitro* or *in vivo* testing in model organisms prior to phage therapy application, either to assess the therapy early on or to select the phages to be used within the phage cocktail. In the specific case of Chan *et al.*, phage cocktail compositions were personalized for each patient based on results from these tests.³¹ This indicates that for phage therapy to be successful, prior assessment of phages through *in vivo* or *in vitro* testing is a prerequisite.

Table 3: Details of Phage Treatment in Studies Involving Phage Therapy. This table summarizes the details of the phage formulations, delivery methods, and dosing strategies used in the reviewed studies.

Authors	Phage Strategy	Route	Approx. Dose	Antibiotics
Li <i>et al.</i> ¹⁸	Single phage treatment, later switched to dual phage	Inhalation	10 ⁶ PFU	Continued
Leitner <i>et al.</i> ¹⁹	Commercial cocktail (Pyophage)	Intravesical	10 ⁴ –10 ⁵ PFU	Standard care
Sarker <i>et al.</i> ²⁰	Commercial cocktails (T4: 11 phages, Microgen ColiProteus: 17 phages)	Oral	~10 ⁵ –10 ⁶ PFU	None
Fedorev <i>et al.</i> ²⁸	Commercial cocktail (4 phages)	Local + injection	≥10 ⁶ PFU/mL	Systemic antibiotics
Kim <i>et al.</i> ²⁹	Cocktail (6 phages)	Intraurethral, IV, oral	10 ⁶ –10 ¹¹ PFU	TMP-SMX
Jault <i>et al.</i> ³⁰	Cocktail (12 phages)	Topical	10 ² –10 ⁶ PFU/mL	Optional
Chan <i>et al.</i> ³¹	Personalized cocktails (2-3 phages)	Inhalation	3 mL × 7 days	Commonly colistin
Gupta <i>et al.</i> ³²	Customized cocktail (20 phages)	Topical	10 ⁶ PFU	Prior antibiotics
Wright <i>et al.</i> ³³	Cocktail (6 phages)	Topical (ear)	10 ⁶ PFU/phage	None
Nick <i>et al.</i> ³⁴	Dual phage	IV	10 ⁶ –10 ⁹ PFU	Concomitant

As seen in Table 3, treatments vary between commercially available phages—such as Pyophage and Microgen ColiP-

roteus^{19, 20}—engineered phages, and customized cocktails, demonstrating the current diversity of available phage therapies.

Many routes of administration were utilized. They included intravenous,^{29,34} respiratory,¹⁸ oral,^{20, 29} or intraurethral,²⁹ but topical treatments—such as intravesical,¹⁹ bone cement,²⁸ ear drops,³³ dressings,³⁰ or direct topical³²—proved to be the most common. The majority of these cases were because topical treatment was simply the most practical administration route, as these studies pertain to burns, wounds, ear infections, etc.

Jault *et al.* do claim that topical treatments could reduce the frequency of adverse effects.³⁰ Despite this, the review found that the frequency of adverse events from studies using topical treatments is still comparable to those that do not.

The dosages and concentrations were also highly varied. They ranged from 10² PFU/mL³⁰ to 10¹² PFU in the urethral treatment from Kim *et al.*²⁹ High doses were often used for systemic or severe infections,^{29,34} while lower or moderate doses were used more frequently in topical treatments.

The majority of studies utilized antibiotics in conjunction with phage therapy, except Sarker *et al.* and Wright *et al.*^{20,33} Jault *et al.* allowed antibiotics at the discretion of the physician, depending on the patient's needs.³⁰ This may have been because of the previously discussed possibility of synergistic activity between bacteriophages and antibiotics. However, when comparing the efficacy data of the trials that employed antibiotics to those that did not, no obvious distinction is found, which makes it unclear how much of an impact the administration of antibiotics actually had. These varied results can be partly attributed to inconsistencies in trial structure.

Regarding the issue of phage therapy producing phage resistance, only Li *et al.* indicated phage resistance occurring in the patient.¹⁸ Li *et al.* was also the only study to treat the patient with a single phage treatment.¹⁸ Phage resistance developed in response to the single phage treatment, but was resolved when the patient switched to a cocktail treatment. The lack of comparable studies limits the generalizability of these results. However, this indicates a possible avenue for future research into phage-resistant infections, and also resolves one of the major drawbacks of phage therapy that was previously discussed.

Additionally, many of the studies using antibiotics in combinations with phages could have worked to prevent resistance, as well as increasing the efficacy of the study, as antibiotics and phages have previously been shown to produce a synergistic effect on each other.¹⁹ However, it also reflects that a high degree of clinical caution is still present.

Lastly, phage therapy is the most successful in cases involving localized, chronic biofilm-associated infections.^{18, 28, 32-34} Most likely, this is because these infections were all localized, not systemic, reducing variability in immune-related clearance. Moreover, they involved biofilms, for which phage therapy has already been demonstrated to be proficient at tackling.²⁴ They also involved fairly predictable pathogens, such as *P. aeruginosa* or *S. aureus*, which allowed for the easy selection of the appropriate phages from a phage bank. All of these aspects align clearly with the predicted behavior of phage therapies based

on their previously discussed advantages and disadvantages, indicating that, as phage therapy grows in commercial popularity, it will most likely be first implemented for infections similar to these: localized, biofilm-associated infections where standard antibiotic regimens have failed.

Current Attitudes Towards Phage Therapy:

There are currently several mixed opinions regarding phage therapy. On one hand, there is currently a steady increase in the number of clinical trials related to phage therapy.²⁵ The focus is usually on therapies targeting high-priority bacteria such as *P. aeruginosa* and *S. aureus*. This creates an optimistic outlook for the future of phage therapy as a more broadly utilized commercial treatment. Currently, though, phage therapy in the United States and Western Europe is normally employed as part of compassionate use cases. These cases are ones involving patients with life-threatening conditions who have not benefited from traditional treatment courses.³⁵ Success of these compassionate use cases^{18, 34} improves the confidence held in phage therapy by researchers and physicians.

Currently, surveys regarding the awareness of phage therapy reveal that both the general public and physicians are not very familiar with phage therapy as a treatment option. However, the study also noted that patients with specific conditions, such as cystic fibrosis, did show strong interest in participating in clinical trials once they were given more information about phage therapies.³⁶

Despite these positive outlooks, there are still many challenges and skepticism that impede the progress of phage therapy. One prominent issue is that of data access. The high specificity of phages means that in order to treat a patient, they need to receive either an extremely broad commercial, fixed treatment or a personalized phage cocktail. In order to personalize the cocktail, the hospital or the pharmacy must have access to a large and diverse bank of phages, as previously mentioned, which is an endeavor that can be expensive and impractical.²⁵ The regulatory hurdles that result from phage therapy's differences from traditional medications also create skepticism about how worthwhile it is to invest time in developing new therapies when there is little existing regulatory framework for treatments of this kind.

■ Conclusion

The recent resurgence of interest in bacteriophage therapy demonstrates the rising need to find alternatives to conventional antibiotics. Based on the reviewed literature and studies, phage therapy exists in a nuanced landscape where regulatory and logistical challenges counterbalance its applications and efficacy.

Based on the compiled clinical studies, phage therapy is particularly effective in situations where conventional antibiotics fail, such as biofilm-associated cases, chronic wounds, and cases involving multi-drug resistant pathogens like *P. aeruginosa*, *S. aureus*, and *K. pneumoniae*.^{18, 28, 29, 32-34} Their tendency to replicate at the infection site and their ability to act synergistically alongside antibiotics also speaks to the benefits of phage ther-

apy and combination approaches over antibiotic courses alone. Adverse effects, if any, were observed to be generally mild, suggesting the treatments' safety when under controlled conditions.

However, the application of phage therapy in clinical settings remains dubious. The narrow host range of phages creates a vast array of problems when trying to formulate effective therapies, as wide-ranging fixed formulas are limited in their potency and personalized therapies require the support of well-constructed phage libraries, along with a significant amount of time and labor. This—along with shaky pharmacokinetics, immunological interactions, and regulatory frameworks that are not yet equipped to handle variable treatments—limits any broader applications of phage therapy that extend beyond compassionate use cases. The high variability among clinical trials regarding trial size, screening processes, and methodology also indicates that while research into phage therapy is growing, the field still remains in its infancy and lacks standardization.

Broader use would require several systemic changes to be made. Clinical trials need to develop standardized protocols that manage dosing, administration routes, and monitoring criteria. This will allow for easier synthesis of data and facilitate regulatory approval. It is also important to expand phage libraries and maintain broad phage repositories. This will improve accessibility, shorten treatment delays, and support more personalized formulas. Combining phages with antibiotics, as many trials have already done, should also become a standard practice, as this will exploit phage-antibiotic synergy to produce more efficient results.

The most critical step for phage therapy is long-term safety studies and continuous monitoring of phage resistance patterns. These are critical for sustainable implementation. Regulatory agencies should also consider introducing approval pathways for phage therapies that accommodate their unique drug characteristics, allowing for the treatment to receive approval without compromising safety. Finally, broader education initiatives targeting both providers and patients can help improve awareness and practical use of phage therapy. All of these initiatives will ensure that phage therapy can be applied effectively and ethically.

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The Hidden Cost of Loneliness: Social vs. Solitary Loneliness

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ABSTRACT: While previous research has focused on understanding loneliness as a solitary experience, I compared the experiences of loneliness, a state of distress or discomfort that results from the perception of a gap between one's desires for social connection and actual experiences of it, while being physically alone (solitary loneliness) versus loneliness while being in the presence of others (social loneliness) across four online experiments ($N = 520$). In these experiments, I randomly assigned participants to one of two conditions: imagine doing an activity with a group of people while feeling lonely (social loneliness) or imagine doing an activity alone while feeling lonely (solitary loneliness). My findings revealed that social loneliness led to greater feelings of negativity, discomfort, and disconnection compared to solitary loneliness. I found similar results across all studies, which focused on different contexts. I theorize that this effect occurs because social loneliness involves comparing oneself to others, which amplifies negative emotions. In contrast, solitary loneliness does not inherently have this comparative element. These findings suggest that addressing the problem of loneliness may not be about increasing social contact alone but rather fostering more intentional and meaningful interactions.

KEYWORDS: Behavioral and Social Sciences, Sociology and Social Psychology, Social Interactions, Loneliness.

■ Introduction

Decades of research has found that loneliness is often perceived as a negative experience. Social isolation, A state in which the individual lacks a sense of belonging socially,¹ and loneliness are often associated with increased risks of morbidities and a greater likelihood of developing high blood pressure.² Loneliness is also often linked to suicidal behaviors. Loneliness is commonly seen as a major factor contributing to mental health issues like depression.³ Loneliness is also closely related to emotional regulation strategies that are isolating.⁴ For example, individuals experiencing stronger feelings of loneliness often suppress their emotions and distance themselves from others, which only increases their sense of isolation. Much research has focused on the negative outcomes of loneliness, as chronic loneliness is linked to a decline in positive social interactions, which can lead to poorer physical health and overall well-being.⁵ Despite extensive research on loneliness, much of it has concentrated on solitary loneliness, or being physically alone.

Research has long emphasized the importance of surrounding oneself with others as a remedy for loneliness.⁶ Social connection is widely recognized as a powerful antidote to loneliness and a key contributor to psychological well-being. Social isolation, by contrast, has been identified as a major risk factor, not only for loneliness but also for adverse health outcomes, including increased risk of premature mortality.⁷ Beyond reducing loneliness, social connection also fosters everyday positive experiences, such as a sense of belonging and even enjoyment from brief interactions with strangers.⁸ Interventions aimed at alleviating loneliness often focus on enhancing social interaction, whether through cognitive and behavioral therapies that help individuals stay present and manage distress,⁹ or

through simple gestures like a smile or short conversation that can increase social awareness and reduce feelings of disconnection.¹⁰ Across these approaches, the common thread is clear: engaging with others remains a central and effective strategy for addressing loneliness.

However, loneliness is not always linked to solitude.¹¹ Social loneliness is a form of loneliness experienced even when surrounded by others.¹² Though greater social engagement is often seen as a solution to loneliness, it can sometimes result in social loneliness instead. Social loneliness and emotional isolation, feeling disconnected or unable to share feelings with others, have many negative effects.¹³ For example, research on students transitioning from high school to university shows high levels of emotional and social loneliness, both associated with depression and anxiety.¹⁴ Social loneliness is also a concern for people of older ages, as loneliness affects their overall health in ways when changes occur, such as retirement, declining health, and reduced mobility, disrupting social interactions.¹⁵

While much research has examined both solitary and social forms of loneliness, the relationship between the two and their distinct consequences has received less attention. This study aims to compare the emotional impact of solitary loneliness (feeling lonely while physically alone) and social loneliness (feeling lonely while in the presence of others), and to determine which elicits more intense negative emotions. I predict that social loneliness will lead to stronger negative feelings than solitary loneliness, potentially due to the presence of social comparison. In social settings, being surrounded by others who appear connected may amplify feelings of disconnection, whereas in solitude, the absence of comparison may buffer against the emotional intensity of loneliness.¹⁶ Being around people who seem more socially connected may make someone

feel more disconnected. My interest in this topic began with a general focus on loneliness. In reviewing the literature, I found that most studies explore loneliness in solitude and consistently link it to negative emotional outcomes. However, I noticed a gap in research examining loneliness that arises within social contexts. This observation led to my central research question: Does loneliness feel worse when one is physically alone, or when one is surrounded by others?

■ Methods

I conducted four experiments. The first and second experiments were run on Prolific, an online research platform, on September 1, 2024. The four experiments had IRB and informed consent from all participants.

For Experiment 1, there were 105 participants (mean age = 40.10 years, SD = 13.75; 55.24% female, 40.95% male, and 3.81% other; 67.62% White or European, 14.29% Black or African American, 9.52% Asian or Asian American, 5.71% Hispanic or Latino/a, and 2.86% other). For Experiment 2, there were 106 participants (mean age = 36.06, SD = 12.07; 56.60% female, 38.68% male, and 4.72% other; 55.66% White or European, 17.92% Black or African American, 12.26% Asian or Asian American, 6.60% Hispanic or Latino/a, and 7.56% other).

In both experiments, I developed two scenarios to test my research question: eating lunch and completing a school project. Each experiment had two conditions: a social loneliness condition (feeling lonely while engaging in the activity with others) and a solitary loneliness condition (feeling lonely while engaging in the activity alone). Participants were randomly assigned to one of the two conditions.

For Experiment 1 (lunch scenario), participants in the social loneliness condition were told: "Imagine you are spending time with a group of people. During lunch, you sit with them, but they are engaged in a conversation that you are not part of. You listen to their discussions, but you don't speak, and no one addresses you directly." Participants in the solitary loneliness condition were told, "Imagine you are spending time on your own. During lunch, you sit by yourself at a table, eating quietly with no one else around. You don't speak, and no one addresses you directly."

For Experiment 2 (school project scenario), participants in the social loneliness condition were told: "Imagine you've just moved into a new school. You're assigned to work on a project with a group of classmates. You try to work with them, but you continue to work quietly by yourself, without interacting with anyone or sharing your ideas." Participants in the solitary loneliness condition were told, "Imagine you've just moved into a new school. You're assigned to work on a project alone. You continue to work quietly by yourself, without interacting with anyone or sharing your ideas."

After reading and imagining each scenario, participants responded to several questions. For valence, which refers to the pleasantness or unpleasantness of an emotional stimulus,¹⁷ they answered: "How would you feel in this scenario?" on a scale from 1 (extremely negative) to 7 (extremely positive). For uncomfortable and discomfort, which refers to not

feeling comfortable and pleasant,¹⁸ they answered: "To what extent would you feel uncomfortable/discomfort in this scenario?" from 1 (not at all) to 7 (very much). For disconnection, which refers to the feeling of being separate from someone or something else and not fitting well together or understanding each other,¹⁹ they answered: "To what extent would you feel disconnected from others in this scenario?" from 1 (not at all) to 7 (very much). Participants then completed demographic questions about age, gender, and race.

To expand my study, I conducted Experiments 3 and 4 on November 25, 2024.

For Experiment 3, there were 155 participants (mean age = 39.86 years, SD = 13.28; 54.19% female, 41.94% male, and 3.87% other; 57.42% White or European, 24.52% Black or African American, 5.81% Asian or Asian American, 5.81% Multiracial, 2.58% Hispanic or Latino/a, and 3.87% other). For Experiment 4, there were 154 participants (mean age = 40.79 years, SD = 14.31; 64.29% female, 33.77% male, and 1.95% other; 64.29% White or European, 21.43% Black or African American, 7.79% Asian or Asian American, 2.60% Hispanic or Latino/a).

These experiments used new scenarios: going to the gym and shopping. For Experiment 3 (gym scenario), participants were randomly assigned to one of two conditions. In the social loneliness condition, they were told: "Imagine you are going to the gym with your gym buddies in the morning. You work out together, but you are not talking to them. You hear your gym buddies checking in with each other's progress, but you remain silent and continue working out without talking to anyone." In the solitary loneliness condition, they were told: "Imagine you are going to the gym alone in the morning. You work out alone, and you are not talking to anyone. You hear others checking in with their progress, but you remain silent and continue working out without talking to anyone."

For Experiment 4 (shopping scenario), participants were randomly assigned to one of two conditions. In the social loneliness condition, they were told: "Imagine you are going to a mall with other people. You browse different stores, picking out items you like. You see the other people you came with shopping and talking, but you remain quiet and continue shopping alone." In the solitary loneliness condition, they were told: "Imagine you are going to a mall alone. You browse different stores, picking out items you like. You see other groups of people shopping and talking, but you remain quiet and continue shopping alone."

After reading and imagining each scenario, participants responded to the same questions from Experiments 1 and 2. Participants then completed demographic questions about age, gender, and race.

After collecting the data, I analyzed them using t-tests in R Studio (Version 2024.04.2+764; R Core Team, 2024). I also used Google Sheets to create bar charts displaying the results for all four studies.

■ Results and Discussion

Experiment 1:

Participants in the social loneliness condition reported significantly more negative valence ($M = 3.12$, $SD = 1.20$, $SE = 0.17$) than those in the solitary loneliness condition ($M = 4.78$, $SD = 1.50$, $SE = 0.21$), $p < .001$. Similarly, participants reported significantly more discomfort in the social loneliness condition ($M = 4.15$, $SD = 1.89$, $SE = 0.26$) than in the solitary condition ($M = 2.29$, $SD = 1.69$, $SE = 0.24$), $p < .001$. Participants also reported feeling more uncomfortable in the social loneliness condition ($M = 4.13$, $SD = 2.04$, $SE = 0.28$) than in the solitary condition ($M = 2.43$, $SD = 1.92$, $SE = 0.27$), $p < .001$. Finally, participants felt significantly more disconnected in the social loneliness condition ($M = 5.10$, $SD = 1.59$, $SE = 0.22$) than in the solitary loneliness condition ($M = 4.06$, $SD = 1.93$, $SE = 0.27$), $p = .004$. In summary, Study 1 demonstrates that participants who imagined experiencing social loneliness reported more negative valence, greater discomfort, and stronger feelings of disconnection than those who imagined solitary loneliness (see Figure 1).

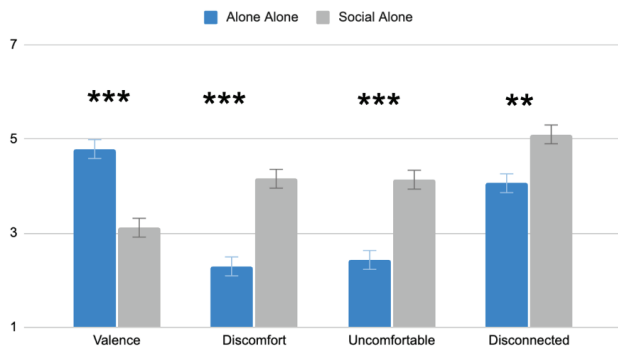


Figure 1: In the lunch scenario, participants in the social loneliness condition reported feeling more negative, uncomfortable, and disconnected than participants in the solitary loneliness condition. The scale ranges from 1, which indicates not at all, to 7, which indicates very much, except for valence, for which 1 indicates extremely negative and 7 indicates extremely positive. (* $p < .05$, ** $p < .01$, *** $p < .001$). Error bars show standard error.

Experiment 2:

Participants in the social loneliness condition reported significantly more negative valence ($M = 3.47$, $SD = 1.31$, $SE = 0.18$) than those in the solitary loneliness condition ($M = 4.08$, $SD = 1.60$, $SE = 0.22$), $p = .036$. Participants also reported greater discomfort in the social loneliness condition ($M = 4.26$, $SD = 1.91$, $SE = 0.26$) than in the solitary condition ($M = 3.31$, $SD = 1.96$, $SE = 0.27$), a statistically significant difference, $p = .013$. Similar results were found for uncomfortability, with higher ratings in the social loneliness condition ($M = 4.30$, $SD = 2.07$, $SE = 0.28$) compared to the solitary condition ($M = 3.30$, $SD = 1.97$, $SE = 0.27$), $p = .013$. Finally, participants felt more disconnected in the social loneliness condition ($M = 5.36$, $SD = 1.52$, $SE = 0.21$) than in the solitary loneliness condition ($M = 4.35$, $SD = 1.78$, $SE = 0.25$), $p = .002$. In summary, Study 2 shows that participants in the social loneliness condition had more negative valence, greater discomfort, and increased feelings of disconnection compared to those in the solitary loneliness condition (see Figure 2).

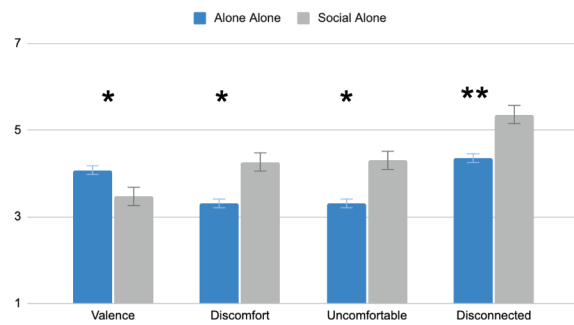


Figure 2: In the school project scenario, participants in the social loneliness condition reported feeling more negative, uncomfortable, and disconnected than participants in the solitary loneliness condition. The scale ranges from 1, which indicates not at all, to 7, which indicates very much, except for valence, for which 1 indicates extremely negative and 7 indicates extremely positive. (* $p < .05$, ** $p < .01$, *** $p < .001$). Error bars show standard error.

Experiment 3:

Participants in the social loneliness condition reported significantly more negative valence ($M = 2.76$, $SD = 0.81$, $SE = 0.09$) than those in the solitary loneliness condition ($M = 3.44$, $SD = 0.95$, $SE = 0.11$), $p < .001$. Participants also reported greater discomfort in the social loneliness condition ($M = 3.25$, $SD = 1.61$, $SE = 0.18$) than in the solitary condition ($M = 2.37$, $SD = 1.49$, $SE = 0.17$), a statistically significant difference, $p < .001$. Similarly, participants reported feeling more uncomfortable in the social loneliness condition ($M = 3.33$, $SD = 1.67$, $SE = 0.19$) than in the solitary condition ($M = 2.46$, $SD = 1.63$, $SE = 0.19$), $p = 0.001$. Finally, participants reported significantly more disconnection in the social loneliness condition ($M = 4.40$, $SD = 1.75$, $SE = 0.20$) compared to the solitary condition ($M = 3.62$, $SD = 2.04$, $SE = 0.23$), $p = 0.012$. In summary, Study 3 demonstrates that participants in the social loneliness condition experienced more negative valence, as well as greater discomfort and disconnection, compared to those in the solitary loneliness condition (see Figure 3).

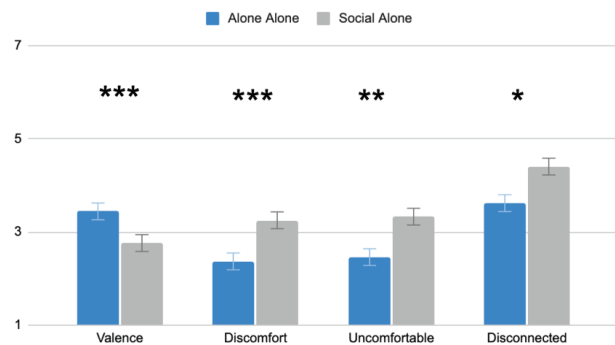


Figure 3: In the gym scenario, participants in the social loneliness condition reported feeling more negative, uncomfortable, and disconnected than participants in the solitary loneliness condition. The scale ranges from 1, which indicates not at all, to 7, which indicates very much, except for valence, for which 1 indicates extremely negative and 7 indicates extremely positive. (* $p < .05$, ** $p < .01$, *** $p < .001$). Error bars show standard error.

Experiment 4

Participants in the social loneliness condition reported significantly more negative valence ($M = 2.97$, $SD = 0.95$, $SE = 0.11$) than those in the solitary loneliness condition ($M = 3.42$, $SD = 0.88$, $SE = 0.10$), $p = 0.003$. Participants also reported

greater discomfort in the social loneliness condition ($M = 3.16$, $SD = 1.50$, $SE = 0.17$) than in the solitary condition ($M = 2.67$, $SD = 1.68$, $SE = 0.19$); however, this difference was not statistically significant, $p = 0.062$. For discomfort, participants again reported higher ratings in the social loneliness condition ($M = 3.23$, $SD = 1.60$, $SE = 0.18$) than in the solitary condition ($M = 2.59$, $SD = 1.63$, $SE = 0.19$), and this difference was statistically significant, $p = 0.017$. Finally, participants reported feeling more disconnected in the social loneliness condition ($M = 4.00$, $SD = 1.80$, $SE = 0.21$) than in the solitary loneliness condition ($M = 3.82$, $SD = 1.91$, $SE = 0.22$), but this difference was not statistically significant, $p = 0.543$. In summary, Study 4 demonstrates that participants in the social loneliness condition experienced more negative valence and greater discomfort, though differences in discomfort and disconnection did not reach statistical significance (see Figure 4).

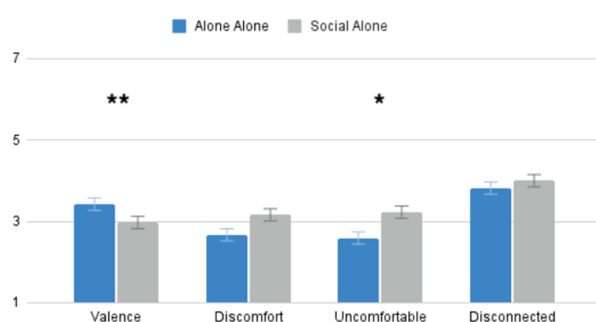


Figure 4: In the shopping scenario, participants in the social loneliness condition reported feeling more negative and uncomfortable than participants in the solitary loneliness condition. The scale ranges from 1, which indicates not at all, to 7, which indicates very much, except for valence, for which 1 indicates extremely negative and 7 indicates extremely positive. (* $p < .05$, ** $p < .01$, *** $p < .001$). Error bars show standard error.

Conclusion

In this research, I explored how people experience two types of loneliness: solitary loneliness (feeling alone when by yourself) and social loneliness (feeling alone even when others are around). Although some results weren't statistically significant, there was a consistent trend across four everyday scenarios: participants reported more discomfort, negative valence, and a stronger sense of disconnection in the social loneliness condition than in the solitary loneliness condition. I theorize that this is because social loneliness involves comparing yourself to others, which may make people feel worse. Further, while this pattern held across all four studies, the strength of the effect varied depending on the scenario. For instance, the emotional valence difference was stronger in some situations (like in Study 1 and Study 3), but smaller in others (like Study 4), possibly because different settings (e.g., eating lunch, going to the gym) bring out different feelings of connection or isolation, or certain activities make social comparison more noticeable. A possible reason is that Study 4's setting involves shopping, which may not evoke immediate or obvious feelings of loneliness, as it is an action often done alone.

Several limitations of my research offer possibilities for future research. To begin, I used a relatively small sample size,

with over 100 participants for each study. The mean ages for the study were between 36 and 40 years old, so the results may not apply as well to younger adults (e.g., 18-30) or older adults (e.g., above 50). Future studies might target different age groups to investigate if people respond differently based on life stage. Further, a key source of variability in the data is individual differences among participants. Participants with different familial, social, or economic backgrounds or varying levels of familiarity with the scenarios may perceive and experience loneliness differently. Future research might consider examining whether and how these differences influence the results.

One main limitation of this research is the hypothetical nature of the scenarios. Rather than experiencing these scenarios, participants were asked to imagine the scenarios in their minds, which introduces potential recall bias.²⁰ This aspect contributes to the challenge of knowing if their self-reported responses would reflect how they would feel in real life. Additionally, because participants self-reported their feelings, their answers could have been influenced by their mood, interpretation of the questions, and/or prior experiences with loneliness. To build on this work, future research could use real-life experiments in which participants engage in these scenarios rather than imagining them. Researchers could also study the long-term effects of social and solitary loneliness, not just how people feel in the moment.

To conclude, loneliness is a widespread experience that is often linked to negative outcomes.²¹ Research suggests that engaging with strangers provides greater psychological benefits than solitude, yet people tend to prefer being alone, mistakenly believing it leads to greater happiness.^{8,22} Studies show that even brief conversations with strangers can enhance well-being,^{8,23} and interactions with weak ties may be more beneficial for emotional health than those with close connections sometimes.²⁴ However, many hesitate to initiate conversations due to fear or discomfort. Loneliness can occur both in solitude and within social settings, with social loneliness—feeling disconnected despite being with others—often evoking stronger negative emotions. My research compared solitary loneliness (feeling lonely when alone) and social loneliness (feeling lonely despite social presence). I found that social loneliness led to greater discomfort and disconnection than solitary loneliness. Although people often assume that being around others prevents loneliness, this research shows that meaningful connection, not just the presence of others, is what matters. Social loneliness can feel even worse than being alone, and understanding this difference may help us better support those who are struggling with connection. A limitation of this study is that the vignette conditions may not have fully separated social loneliness and solitary loneliness. Although the social loneliness condition represents the feeling of loneliness in the presence of others, it may also imply social exclusion if participants imagined being around those they are familiar with. Similarly, the solitary loneliness condition may have caused the feeling of being alone rather than loneliness, as actions, such as studying and shopping, often occur alone without feelings of loneliness. Future research should conduct experiments that explicitly state participants felt an unmet desire for social connection,

and can conduct experiments could involve two conditions for “social loneliness,” where one is with friends, and the other is with strangers, to examine if there will be a possible difference. Future research can also combine the measures of interest in discomfort and uncomfortable, as they correlate strongly. Future research can focus on the impact of social comparison on loneliness to provide a better understanding of the connection between the two by including self-report measures of social comparison and testing whether comparison mediates or intensifies loneliness.

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Machine Learning-Driven Meta-Analysis Reveals Shared B Cell Repertoires linked to Multiple Sclerosis

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ABSTRACT: Multiple sclerosis (MS) is an autoimmune, neurodegenerative disease where the immune system damages myelin sheaths in the central nervous system. MS pathogenesis was attributed to auto-reactive T cells. However, recent studies suggest that B cells also play a vital role, including antibody-dependent and independent effects in compartmentalized inflammation. Clonotypes are groups of B cells with the same receptor. To explore the role of B cell clonotypes in MS pathogenesis, we conducted a meta-analysis of raw high-throughput sequencing data of the B cell repertoire of 82 subjects (48 MS patients and 34 controls). A novel combination of density-based clustering (DBSCAN) and supervised ML (random forest) was applied to cluster clonotypes into unique clonotype groups (UCG), and their disease relevance was assessed. Random forest feature importances, Mann-Whitney tests, and empirical power estimation revealed six significant, potentially pathogenic UCGs. Additionally, tertiary analyses revealed genetic variability and clonal expansion as hallmarks of MS-associated B cells. Interestingly, new genomic motifs among our potentially pathogenic UCGs aligned with known IGHV4 family biases. Overall, the study highlights the utility of machine learning analysis in decoding immune repertoires and contributes to our understanding of B-cell involvement in MS pathogenesis, paving the way for future therapeutic exploration.

KEYWORDS: Biology, Computational Biology and Bioinformatics, Multiple Sclerosis, Pathogenic B cells, Machine Learning.

Introduction

Multiple sclerosis (MS) is a chronic, demyelinating disease affecting the body's central nervous system. Currently incurable, the estimate for global MS prevalence is around ~2.8 million people.¹ Symptoms include a lack of coordination, partial or complete loss of vision, fatigue, trouble with memory, and more.² While MS is a B- and T-cell-mediated disease, and B-cell-targeting therapies have demonstrated clinical success, specific pathogenic B-cell clonotypes and BCR sequence patterns that drive disease pathogenesis remain underexplored in current research.³

B cells or lymphocytes are key cells of the adaptive immune system. Primary functions of B cells are antibody production, immunological memory, antigen presentation, and cytokine secretion. Antigen binding to B cell receptors (BCRs) and subsequent activation by helper T cells lead to B cells undergoing clonal expansion, where they generate genetically identical clones. Clonotypes are groups of genetically identical B cells. B cells also offer antigen recognition diversity by undergoing somatic hypermutation. The BCR is a 'fingerprint' for B cells, which provides representative information of the B cell for classification and genetic analyses. The V(D)J regions of the immunoglobulin gene shuffle to generate genetic diversity amongst the BCR.

In multiple sclerosis, research suggests autoantibodies targeting self-antigens are contributors to MS pathogenesis;³ however, it is unclear which B cells are associated with the disease. Anti-CD20 therapies have been proven effective in the

treatment of multiple sclerosis,⁴ further rationalizing the additional exploration of B cells as drug targets.

Machine Learning (ML), which is a subset of artificial intelligence, excels at extracting features from high-dimensional BCR genetic data for clustering and identifying non-linear patterns indicative of pathogenicity that might be missed by conventional analyses. In this study, we performed an ML-driven meta-analysis of the B cell receptor sequencing repertoire of MS patients and healthy controls (HCs) to identify potentially pathogenic B cells for therapeutic targets.

Methods

Figure 1 is a graphical representation of our methodology.

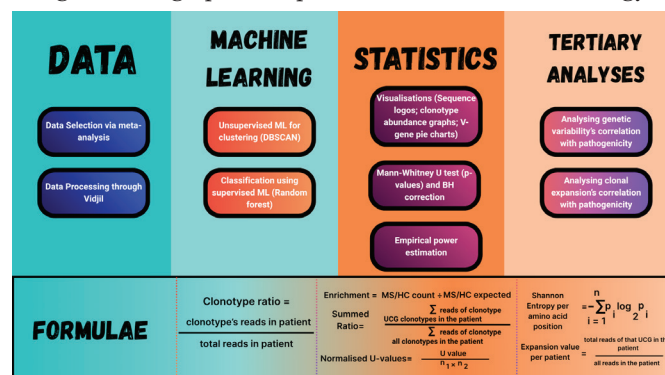


Figure 1: Graphical representation of our methodology; chronology: left to right. Highlights steps for data processing, machine learning, statistics, and tertiary analyses. All formulae used are summarized here.

Dataset & Data Selection:

A traditional limiting factor for RNA-seq meta-analysis studies of this type is the high cost and effort associated with large datasets;⁵ nevertheless, combining datasets of existing studies can eliminate this constraint. This study incorporates a dataset of MS patients larger than any prior study of this nature reviewed by us.

The meta-analysis is performed using diverse search criteria on research databases (such as Google Scholar), including “BCR RNA-seq (MS/HC),” “Immunoglobulin RepSeq (MS/HC),” “AIRR seq (MS/HC),” and “Effect of [MS immune drug] on BCR RNA-seq repertoire of MS patients.” Out of the 8 identified datasets, 2 were exclusively HC datasets,^{6,7} 5 were MS datasets,^{8–12} and 1 was mixed.¹³ A total of 82 subjects (48 MS patients and 34 HCs) were identified for processing.

Data Processing:

Vidjil is a platform for clonal analysis of lymphocyte high-throughput sequencing data of the immunoglobulin region. Downloaded FastQ files from the Sequence Read Archive were uploaded to the Vidjil web server for clonotype analysis. Resultant CSV files provided information regarding each clonotype’s member count (or ‘reads’) and the ratio between the clonotype’s reads in the patient and the total reads of all clonotypes in that patient. CSV files were merged, and unproductive clonotypes (such as those with stop codons in the nucleotide junction) were filtered out. Irrelevant data columns were removed. For patients whose sequencing was performed across various samples, clonotype reads were summed across samples. A new source column was added to specify the patient of origin. Refer to the supplementary manuscript file under ‘S1’ for more details.

Clustering:

We identify thousands of clonotypes, although each clonotype has low frequency counts (reads). This is because B cells undergo somatic hypermutation, where they rapidly mutate their DNA in an immune response. This creates a diverse repertoire of similar B cells but of distinct clonotypes. Consequently, utilizing supervised ML classification on our raw clonotype data is ineffective, as each clonotype becomes a feature for the ML algorithm, and a large number of features with very low variance (most clonotypes have close to 0 reads) massively impacts model performance. Therefore, to counter the high-dimensional clonotype makeup, we cluster similar clonotypes into unique clonotype groups (UCGs); these UCGs are used downstream as our features for classification.

To cluster clonotypes into UCGs, we needed parameters to classify similar clonotypes. We formulate these conditions based on a letter in Leukemia and use them to develop a novel, custom distance function to compare the similarity between two clonotypes.¹⁴ The distances were integrated with density-based clustering. Parameters for similarity are as follows: two clonotypes are in the same cluster when they have a minimum 80% identity in their CDR3 amino acid sequence, similar CDR3 nucleotide sequence (junction) lengths, and the same V gene. We calculated percent identity using the Needle-

man-Wunsch algorithm, implemented through the Biopython library in Python, to align the amino acid sequences. Then, we developed the distance function, which heavily penalizes the distance between clonotypes with a different V gene or with a difference in length of the AA sequence greater than 3. The combination of these three criteria provided a robust, novel metric to compute distances in “biological” space between two clonotypes.

We ran this algorithm on approximately 36 million clonotype pairs $[(\sim 8000 \times \sim 8000) \div 2]$ to create a distance matrix. The matrix was a clonotype X clonotype file, providing a distance score for each comparison between 2 clonotypes. A distance of 0 means the clonotypes are identical; as the difference between the clonotypes increases, so does the distance. We used the DBSCAN clustering algorithm (an unsupervised ML algorithm) to cluster similar clonotypes into UCGs. We use DBSCAN as it is density-based and is compatible with non-standard distance functions, while alternatives (like k-means and k-medians) might require incompatible metrics such as Euclidean distance. Additionally, DBSCAN does not require us to define the number of clusters beforehand.

The clustered UCGs were transformed into a patient X UCGs matrix. In each cell, the ratio of the UCG for that patient is calculated using this formula:

$$\text{ratio} = \frac{\text{reads of UCG in that patient}}{\text{reads of all UCGs in that patient}}$$

Equation 1: Abundance ratio formula.

We use ratios instead of raw reads to normalize for potential study-based differences in sequence preparation (such as bulk versus single-cell sequencers or differing tissue origins). Further details for clustering parameters can be found in the supplementary manuscript file under ‘S2’ and ‘S3’.

Machine Learning to Identify Potentially Pathogenic UCGs:

We devise an ML classification approach to assign weights to our features (UCGs). We use supervised ML algorithms such as Support Vector Machine (SVM), Random Forest, Decision Tree, Logistic Regression, and LightGBM to classify between MS and HC. Supervised ML predicts labels pre-defined by the user (in our case, MS and HC).¹⁵ A stratified train-test split was run with three-fold cross-validation to ensure robust and stable results during hyperparameter tuning and to account for class imbalance. We generate a detailed classification report with performance scores for each model. A confusion matrix is generated for the highest-performing model (Random Forest).

Feature importances from the best-performing model are extracted. Higher importances relative to the other features entail that the UCG was highly influential to the model’s classification decisions. The UCGs with the top 10 feature importances were shortlisted for further analysis. Specifications for classification can be found in section ‘S4’ of the supplementary manuscript file.

Visualizations:

Within each UCG, each B cell clonotype had a corresponding CDR3 amino acid sequence. Sequence logos for each UCG (representative of their clonotype CDR3 makeup) and consensus sequences were generated by i) aligning CDR3 sequences using MUSCLE,¹⁶ ii) extracting sequences from generated FastAs using SeqIO from Biopython, and iii) generating sequence logos and consensus sequences using the logomaker module (see section 'S5' of the supplementary manuscript file). We identify the common parts of the V gene makeup in each UCG. Distribution pie charts for the clonotypes' V gene family, subgroup, and position were generated. Per UCG, we generate an enrichment vs observed bar graph. Values above 1 indicate that the class (healthy or MS) is over-represented in the cluster, values equal to 1 indicate that the class is expressed as expected, and values lower than 1 indicate that it is under-represented. To calculate this, we first calculate the expected count for each class and then calculate the enrichment value.

$$\text{A Expected Count}_{\text{MS or HC}} = (\text{total clonotypes in UCG}) \times \frac{\text{total MS or HC clonotypes}}{\text{total clonotypes overall}}$$

$$\text{B Enriched} = \frac{\text{MS/HC count}}{\text{MS/HC expected}}$$

Equation 2: Enrichment calculation: A. Expected count calculation, B. Enriched score calculation.

For each patient in which the UCG is detected, we generate a summed clonotype ratios histogram. Patients are color-coded in accordance with their disease status.

$$\text{summed ratio} = \frac{\sum_{\text{UCG clonotypes in the patient}} \text{reads of clonotype}}{\sum_{\text{all clonotypes in the UCG}} \text{reads of clonotype}}$$

Equation 3: Summed ratio formula.

Statistical Analysis:

A non-parametric Mann-Whitney U test is performed on the summed ratios for each MS patient in the UCG and those for each HC patient in the UCG. Empirical power estimation with 10,000 resamples is run for each test. Benjamini-Hochberg adjusted p-values are calculated for each UCG to adjust for false positives; these values adjust the raw p-values based on the number of hypotheses (in this case, the top 10 UCGs being tested for significance). UCGs with a p-value < 0.05, a power estimation between 0.800 and 1.000, and a Benjamini-Hochberg adjusted p-value < 0.05 are considered statistically significant. U-values from the Mann-Whitney test are extrapolated and normalized using this formula:

$$\text{normalized U} = \frac{U \text{ value}}{n_1 \times n_2}$$

Equation 4: U-value normalization.

Here, n_1 and n_2 are the sizes of the MS and HC groups. Normalized U values ≥ 0 and ≤ 0.5 indicate HC skew, normalized U values = 0.5 show balanced data, and normalized U values ≥ 0.5 and ≤ 1.0 demonstrate an MS skew. Additional information can be found in section 'S6' of the supplementary manuscript file.

Tertiary Analysis:

Genetic Variability:

To understand whether genetic variability is correlated with UCG pathogenicity, UCGs with the top 20 feature importances and the bottom 20 feature importances with available sequence data were selected for comparison. Shannon entropy per position for the aligned CDR3 sequences per UCG was calculated using this formula:

$$\text{Entropy} = - \sum_{i=1}^n p_i \log_2 p_i$$

Equation 5: Shannon entropy formula.

Here, i is indexing over all amino acids at that position and p_i is the probability (frequency) of the amino acid 'i' being at that position. Shannon entropy is averaged across positions for each UCG. A Mann-Whitney U test is performed on the mean entropies of UCGs with high importances against those of UCGs with low importances. The results are illustrated on a box plot along with indicators of statistical significance (e.g., asterisks based on the Mann-Whitney p-value).

Clonal Expansion:

In addition to genetic variability, we wanted to learn whether clonal expansion in MS patients is correlated with UCG pathogenicity. Once more, UCGs with the top 20 and bottom 20 feature importances with available sequence data were chosen for comparison. Clonal expansion per MS patient was calculated as follows:

$$\text{Expansion}_{\text{patient}} = \frac{\text{Total reads of that UCG in that patient}}{\text{All reads in that patient}}$$

Equation 6: Expansion formula.

Expansion values for all MS patients are averaged per UCG. A Mann-Whitney U test is performed on the mean expansion values for the highest-performing UCGs against those of the lowest-performing UCGs.

Results

Segregation in Principal Component Analysis (PCA) Between Healthy and Diseased Classes:

A qualitative analysis of the PCA plots reveals a promising segregation between the healthy and diseased classes, indicating a meaningful difference for classification (Figure 2). The relative homogeneity of data from different studies indicates little to no source bias. PC1, PC2, and PC3 explained 29.68%, 16.74%, and 6.51% of the total variance, respectively, based on their associated eigenvalues. They cumulatively account for 52.94% of the variance.

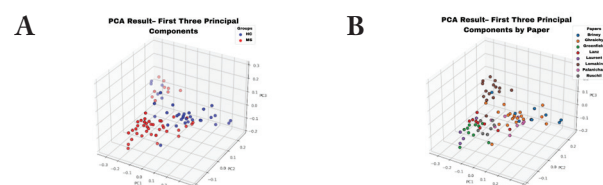


Figure 2: Principal Component Analysis (PCA) and ML accuracy. A. PCA colored by disease status shows clear separation between healthy and diseased classes. B. PCA colored by paper shows limited source-wise separation.

High ML accuracy during classification using Random Forest:

The model with the highest weighted average F1-score was 87.79%; this was achieved by the random forest model. The confusion matrix for the random forest model (Figure 3) reveals 0 true negatives and only 2 false positives.

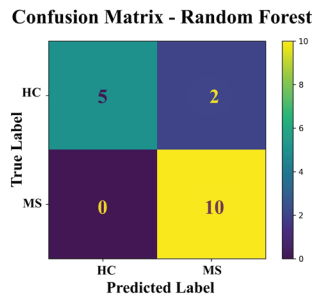


Figure 3: The random forest model confusion matrix. Outlines classification performance, showing 5 correctly predicted HC, 2 HC misclassified as MS, 0 MS misclassified as HC, and 10 correctly predicted MS.

Identification of Statistically Significant UCGs:

The classification model was trained on our unique clonotype groups – UCGs that contributed more to the model’s decision between healthy and diseased were assigned higher feature importances. We extracted these importances to rank our UCGs. Figure 4 represents the importance of the 20 UCGs with the highest feature importances.

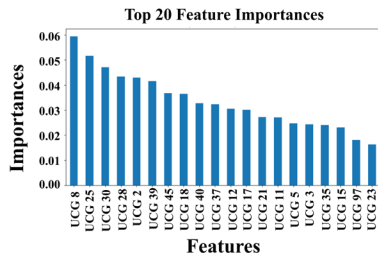


Figure 4: Top 20 feature importances extracted from the random forest model.

Table 1 summarizes the feature importances, Mann-Whitney p-values, Benjamini-Hochberg corrected p-values, and empirical power estimation values for the UCGs with the top 10 importance values.

Table 1: Comparison table of UCGs with top 10 feature importances. Statistically significant UCGs have a p-value < 0.05, an empirical power estimation >0.8, and a corrected p-value < 0.05. Statistically significant UCGs are highlighted in green.

UCG Designation	Feature Importance	Mann-Whitney p-value (<0.05 is significant)	Empirical Power Estimation Value (>0.8 is significant)	Benjamini-Hochberg p-value (<0.05 is significant)
UCG 8	0.0595	<0.0001	1.000	<0.0001
UCG 25	0.0516	0.0050	0.952	0.0125
UCG 30	0.0471	0.3114	0.161	0.3460
UCG 28	0.0434	0.4140	0.001	0.4140
UCG 2	0.0430	0.0002	0.989	0.0007
UCG 39	0.0417	<0.0001	1.000	<0.0001
UCG 45	0.0367	0.0068	0.822	0.0127
UCG 18	0.0366	0.0076	0.801	0.0127
UCG 40	0.0327	0.1730	0.263	0.2163
UCG 37	0.0324	0.1085	0.362	0.1550

Mann-Whitney p-values (and Benjamini-Hochberg corrected p-values) are calculated from the summed ratios for the MS and HC classes in each UCG. Empirical power estimation validates the accuracy of the p-value. From this table, we extrapolate 6 statistically significant and potentially pathogenic UCGs.

Tertiary Analyses Reveal Genetic Variability and Clonal Expansion as Markers for Pathogenic Clonotype Groups:

Box plots are generated from the mean Shannon entropy scores and mean expansion scores from the best- and worst-performing UCGs alongside statistical analyses. The genetic variability box plot (Figure 5a) reveals a statistically significant difference (p-value = 0.0004 [<0.0005]) between the mean Shannon entropy from potentially pathogenic UCGs and those with low feature importances. Comparing medians shows increased variability in the top 20 UCGs. In the clonal expansion box plot (Figure 5b), the median comparison and low Mann-Whitney p-value suggest that clonal expansion in MS patients is positively correlated with disease-causing effects.

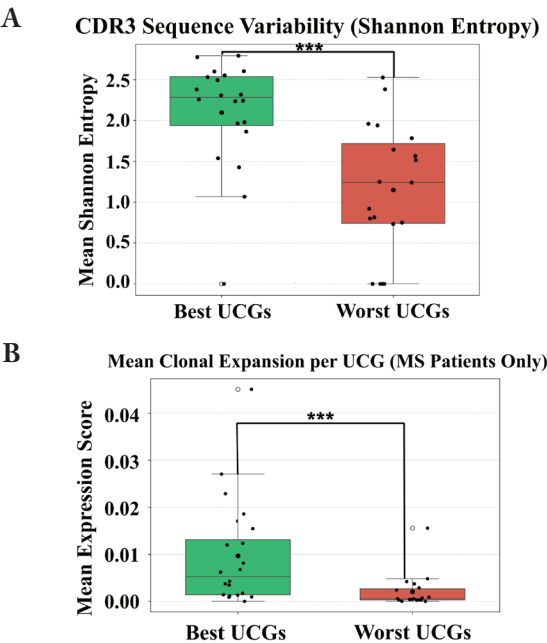


Figure 5: Tertiary analysis box plots comparing potentially pathogenic UCGs and those with low feature importances. A. The CDR3 sequence variability comparison plot. The green box plot represents the mean Shannon entropy for the top 20 UCGs, while the red box plot displays the same for the bottom 20 UCGs. A p-value of 0.0004 indicates a statistically significant difference between the two classes. B. The mean expansion values comparison plot. The green plot represents the top 20 UCGs, and the red plot represents the bottom 20 UCGs. A p-value of 0.0008 suggests a statistically significant difference between the data. A comparison of the medians reveals higher MS clonal expansion in the top 20 UCGs.

Per-UCG Visualizations Display Genetic Characteristics and Abundance of Each UCG:

Visualizations summarizing the relevant genetic characteristics of each UCG were generated. Figure 6 is a graphical representation of the components of these UCG visualizations.

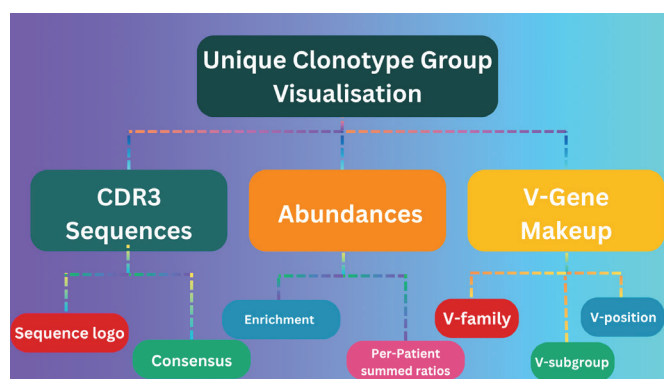


Figure 6: UCG visualization flow. A UCG visualization contains CDR3 sequence, UCG abundance, and V-gene makeup details.

The per-UCG visualization includes a sequence logo, an enrichment bar graph, a summed ratio (abundance) histogram, and V gene distribution pie charts. An example of such a visualization is seen below (Figure 7).

The sequence logo, consensus sequence, and V-gene charts can tell us information about the genetic makeup of the clonotype group. While both the enrichment bar graph and the summed ratios histogram display clonotype makeup, the noteworthy difference is that the bar graph simply uses the frequency (count) of healthy and MS clonotypes in its calculations, while the histogram takes into account the abundance (reads) of individual clonotypes. Importantly, the graphs are not displaying raw counts or reads; rather, they are displaying normalized values (as outlined in methods).

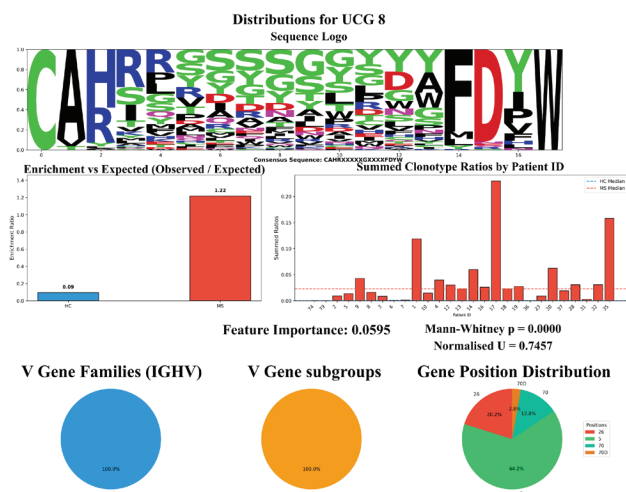


Figure 7: An example of a per-UCG visualization: UCG 8. The sequence logo represents conserved amino acids at each position across clonotypes. The enrichment bar graph presents the disease distribution of the overall clonotype makeup. The summed ratios represent UCG abundance per patient. The V gene pie charts display the V-gene makeup for the UCG. The Mann-Whitney p-value, normalized U value, and feature importance are displayed.

Among the six statistically significant UCGs, several genomic and abundance patterns emerged. UCG 8 demonstrated strong MS enrichment, with prominent clonal expansion in several MS patients; additionally, it is IGHV2-associated. UCG 25 is the most MS-exclusive UCG, with negligible HC enrichment. Similarly, UCG 2 and 39 possess a strong MS skew. UCG 45 is unique in that its HC enrichment ex-

ceeds MS enrichment despite higher MS abundance; it has a dominant IGHV4 signature. UCG 18 has an MS-associated abundance pattern. Overall, multiple UCGs showed conserved terminal motifs, variable CDR3 regions, and evidence of MS-associated clonal expansion.

Table 2 summarizes the data from these visualizations for the significant UCGs.

Table 2: Comparison table of the characteristics of the statistically significant UCGs. Refer to citation 5 for corroboration.⁵

UCG	Sequence logo variability	Enrichment skew	U-value skew (<0.5 = HC; >0.5=MS)	Genomic family and subgroup	Gene position(s)
8	Variable	MS	MS	IGHV2	5, 26, 70D, 70
25	Variable	MS	MS	IGHV1	2, 24, 3, 46, 69, 69D, 8
2	Variable	MS	MS	IGHV5	51, 10-1
39	Variable	MS	MS	IGHV1	18, 2, 24, 3, 46, 69, 69D, 8
45*	Variable	HC	MS	IGHV4	30-4, 31, 34, 38-2, 39, 4, 59, 61
18	Variable	MS	MS	IGHV5	51, 10-1

Discussion

Through a machine learning-mediated meta-analysis of B cell sequencing data paired with various statistical analyses, we discovered statistically significant characteristics of MS clonotype groups: genetic variability, through the amino acid sequence logos; clonal expansion, using normalized clonotype reads; and positional genomic data, such as the V region pie charts. To discuss the suitability of the supervised ML approach, we analyzed the PCA graphs, ML scores, and the confusion matrix.

First, by examining the 3D plot from Principal Component Analysis (PCA) (Figure 2), we visualized a separation between the healthy and diseased classes: an encouraging preliminary observation that stipulates a difference in the sequencing repertoire of the two classes that could be subject to further investigation through classification ML.

Next, the high f1-score of 87.79% and high confusion matrix-derived accuracy bolster the significance and reliability of the feature weights extracted from the random forest model and further exemplify that BCR sequencing data, and by extension, B cells, are a suitable source of data to investigate the cause of multiple sclerosis.

Understanding the broader characteristics shared by pathogenic UCGs is one that holds merit in drug design. Our tertiary analysis focused on sequence variability and clonal expansion as characteristics of pathogenic UCGs. In this section, we discussed their viability as candidates for further research based on our statistical tests on their significance in MS pathogenesis.

In order to ascertain the importance of sequence variability in MS pathogenesis, we compared the classes of the impactful UCGs with those of low importance. The low p-value of 0.0004 indicated that there is a statistically significant difference between the CDR3 sequence variability of the two classes. By comparing medians, we observed that an increase in potential pathogenicity is positively correlated with increased

sequence variability. We hypothesized that this increased variability is a result of somatic hypermutation in diseased B cells, a characteristic which has been previously observed in the CSF of MS patients.¹⁷ B cells may undergo somatic hypermutation to refine their BCRs and, by consequence, their autoantibodies, to increase affinity for the body's myelin sheath; this could significantly increase their pathogenic success. Accordingly, we can say that sequence variability is an important characteristic of pathogenic UCGs, and its role and cause in MS pathogenesis should be further investigated in future studies.

With clonal expansion, once more, we observed a significant p-value of 0.0008. Combined with a median comparison, this p-value allowed us to conclude that potentially pathogenic B cells showed a statistically significant increase in clonal expansion in contrast to those with lower feature importances. This is concurrent with research in the past, where increased clonal expansion for B cells in the CSF has been reported in MS patients.¹⁸ We hypothesized that the increase in potentially pathogenic B cells from clonal expansion could exacerbate MS-consequent neurodegeneration, and, therefore, clonal expansion is another crucial characteristic of interest for further investigation.

Ultimately, based on this tertiary analysis, we believe that UCGs displaying increased MS clonal expansion and increased genetic variability should be flagged as those of interest for research on future therapeutic targets; additionally, MS clonal expansion and genetic variability can be identified as global characteristics of potentially pathogenic MS B cell unique clonotype groups. Research on identifying or disproving causal links between these global characteristics and MS pathogenesis is required.

Lastly, we conducted an in-depth analysis of the visualizations of our significant UCGs. From Table 2, we can see that these potentially pathogenic UCGs all mostly have a skew towards MS patients, except for UCG 45, which has a higher HC enrichment score. This is because the enrichment score is based on unique clonotype frequency counts, and we hypothesized that once the MS antigen is encountered, clonal expansion (increasing clonotype abundance) may be prioritized over making unique clonotypes through somatic hypermutation, decreasing the frequency count. This may explain why, while the MS enrichment score in UCG 45 may have been reduced, the total MS clonotype abundance is more significant (indicated by the U-value >0.5). Further investigation is warranted for this observation.

Previous research has demonstrated a VH4 bias in MS B cell clonotypes;⁵ notice that, accordingly, UCG 45 has a VH4 region majority. Moreover, UCG 45 incorporates 5 VH4 regions, which have previously been associated with MS B cells in the CSF (IGHV4-31, IGHV4-34, VH4-39, IGHV4-59, and IGHV4-61), along with 3 other new gene positions. This established a strong confidence in the validity of our pipeline. Additionally, we observed a motif for certain parts of IGHV1 (IGHV1-2, 24, 3, 46, 69, 69D, 8) and IGHV5 (IGHV5-51 and 10-1) across UCGs, which highlighted their importance as regions of interest in future therapeutic target studies. Finally,

we also identified parts of the IGHV2 gene that may be of interest in pathogenicity studies.

■ Conclusion

This study presented a novel approach to identify potentially pathogenic B cell clonotypes using an ML-driven meta-analysis of high-throughput sequencing data, and we identified six UCGs of this nature correlated with MS for future therapeutic research. Through a combination of supervised and unsupervised ML, we identified the motifs ('global characteristics') of genetic variability and clonal expansion among these UCGs: traits of interest for future research on the role of B cells in MS pathogenesis. Our findings exemplify the utility of applying machine learning to immune sequencing data to extrapolate meaningful conclusions from complex, high-dimensional sequences. Corroboration with previous studies confirms the merit and biological relevance of our results, revealing parallels with previously reported IGHV4 family bias. The recurrent clonotype architectures and IGHV-associated patterns we identify in this study could serve as biomarkers for immune repertoire-based MS profiling. Our observed links between MS pathogenicity, clonal expansion, and sequence variability may help identify B-cell subgroups of interest for future therapeutic targeting. In conclusion, our study provides insight into B-cell involvement in MS pathogenesis and creates a novel computational framework for future studies analyzing the MS immune repertoire.

■ Limitations and Future Steps

Our study is strengthened by our comparatively larger sample size than previous MS immune sequencing studies; however, correlating variables such as tissue origin and disease stage with B cells can produce valuable conclusions in the context of disease progression. Future research should prioritize further analysis into factors such as sequencing depth, age/sex, and tissue origin to discover important new insights about the role of B cells in MS pathogenesis while using rigorous normalization to limit the batch effect arising from data heterogeneity. Clinical verification and analysis of computational results are the next steps in developing B-cell-targeting therapeutics against MS.

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Perceptions of Artificial Intelligence–Based Psychotherapy: A Mixed-Methods Study

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ABSTRACT: Artificial intelligence (AI) has increasingly been integrated into various domains, including psychotherapy. Individuals' perceptions of AI play a critical role in shaping the acceptance, perceived effectiveness, and trustworthiness of AI-supported therapeutic processes. Given the limited number of studies on this topic, public perceptions of AI-based psychotherapy remain insufficiently explored. This mixed-methods study examined attitudes toward AI-based psychotherapies. In the quantitative phase, 317 adults aged 18–65 ($M = 41.52$, $SD = 12.30$) completed the Attitudes Toward AI-Based Psychotherapy Scale (ATAIP), the AI Anxiety Scale, and the Attitudes Toward Artificial Intelligence Scale. In the qualitative phase, semi-structured interviews were conducted with 10 participants. Overall, 42.0% of respondents were “unsure” about preferring an AI therapist, and 56.8% reported greater trust in human therapists regarding data security. Correlational analyses showed that attitudes toward AI-based psychotherapy were negatively associated with learning anxiety and AI configuration anxiety, and positively associated with general attitudes toward AI. Qualitative findings indicated that AI-based therapy offers advantages such as low cost, time efficiency, easy accessibility, large-scale data processing, reduced fear of judgment, and reduced human bias. However, concerns emerged regarding data security, information accuracy, lack of face-to-face interaction, and limited emotional authenticity. Overall, participants perceived AI-supported psychotherapy as practically useful yet emotionally limited.

KEYWORDS: Social and Behavioral Science, Clinical Psychology, Artificial Intelligence, Psychotherapy, Perception.

■ Introduction

In recent years, digitalization has increasingly influenced all aspects of daily life, including mental health services, which are undergoing a notable transformation. Systems that can provide support independent of time and place have been developed, thereby improving accessibility and contributing to new models of service delivery.¹ One reason for the rapid expansion of artificial intelligence (AI)–supported systems is the global burden posed by psychiatric disorders, which constitute a major source of morbidity worldwide. Managing these conditions is especially challenging in low-resource settings, where access to mental health professionals is limited. AI has therefore emerged as a promising technology offering innovative and feasible solutions for the diagnosis and management of such disorders.¹

Pioneering studies have demonstrated that AI-based therapeutic tools may yield psychological benefits within relatively short periods. For example, Woebot, a system grounded in Cognitive Behavioral Therapy (CBT), has been shown to reduce depression and anxiety symptoms among young adults.² Similarly, a systematic review by Li *et al.*³ found that AI-based chatbots provide therapeutic benefits across various modalities, including CBT, Acceptance and Commitment Therapy (ACT), and skills-training programs. Additional evidence indicates that chatbot-delivered psychotherapy supports symptom improvement,⁴ and interventions such as Vivibot have reduced anxiety among young adults following cancer treatment.⁵ Moreover, interacting with an empathy-based chatbot after periods of social isolation has been shown to en-

hance mood.⁶ Collectively, these results highlight the potential of AI-supported conversational systems to promote emotional well-being.

Despite these promising outcomes, several studies have emphasized the ethical, emotional, and technical risks associated with AI-based psychotherapy. Users frequently express concerns regarding privacy, data security, confidentiality, lack of human contact, and insufficient empathy.¹ For instance, Aktan *et al.*⁷ reported that participants overwhelmingly trusted human therapists more than AI systems when sharing personal information, with only 14% expressing trust in AI-based psychotherapy. This underscores the continued importance of the human element in therapeutic relationships. Existing literature suggests that while individuals perceive certain advantages of AI-supported psychotherapy—such as enhanced privacy, reduced fear of judgment, 24/7 accessibility, and lower cost—they also identify significant limitations, including reduced empathy, limited human understanding, ethical ambiguities, and data privacy concerns.^{7,8}

Recent findings further indicate that individuals' general attitudes toward AI and their levels of AI-related anxiety play an important role in shaping their acceptance of AI-based therapeutic systems. For example, Keung and So⁹ found that individuals with frequent exposure to AI may develop more negative attitudes toward it during emotionally intense experiences, which in turn influences their preferences for psychological support. Similarly, Wagner and Schwind¹⁰ showed that therapists' AI-related anxieties are directly associated with their intentions to use such systems, and Cecil *et al.*¹¹ reported

a negative association between AI anxiety and technology-use intentions.

Taken together, the existing literature indicates that AI-supported psychotherapy systems possess both strengths and limitations; however, empirical research in this area remains scarce. The present study aims to evaluate perceptions of AI-based psychotherapy, examine ethical, emotional, and technical factors influencing these perceptions, and assess overall acceptability. By integrating quantitative and qualitative methods, the study seeks to provide a comprehensive and nuanced understanding of individuals' perceptions. The quantitative phase investigates associations between AI anxiety, general attitudes toward AI, and attitudes toward AI-supported psychotherapy. In contrast, the qualitative phase explores participants' in-depth evaluations and lived perspectives through semi-structured interviews.

■ Methods

This study employed a mixed-methods design. Eligibility criteria for participation were: (1) being between 18 and 65 years of age, and (2) being a citizen of Turkey. In the quantitative phase, data were collected from 321 individuals; however, four participants were excluded for not meeting the age criterion. The final sample consisted of 317 adults aged 18–65. The following instruments were used in the data collection process:

1. Attitudes Toward AI-Based Psychotherapy Scale (Aktan *et al.*;⁷ ATAIP): This 6-item, 5-point Likert-type scale assesses attitudes toward AI-supported psychotherapy. Higher scores indicate more favorable attitudes (e.g., “AI-based psychotherapy systems can empathize”). In the present study, Cronbach's α was .85.

2. AI Anxiety Scale (Wang & Wang;¹² Turkish adaptation: Terzi¹³): This 21-item, 7-point Likert-type measure assesses anxiety related to artificial intelligence. Higher scores reflect higher levels of anxiety. The scale includes four subdimensions: Learning anxiety (e.g., “Learning to use AI techniques/products makes me anxious.”), Job-replacement anxiety (e.g., “I am afraid that an AI technique/product may make us dependent.”), Sociotechnical blindness (e.g., “I am afraid that an AI technique/product may be misused.”), Structural/physical appearance anxiety (e.g., “I find humanoid AI techniques/products (e.g., humanoid robots) scary.”). In the present study, Cronbach's α was .95.

3. Attitudes Toward Artificial Intelligence Scale (Grassini;¹⁴ Turkish adaptation: Arslan *et al.*¹⁵): This single-factor, 4-item instrument uses a 10-point Likert format. Higher scores indicate more positive attitudes toward AI and greater acceptance (e.g., “I believe that AI will improve my life.”). In the present study, Cronbach's α was .93.

In the qualitative phase, semi-structured interviews were conducted with 10 participants aged 18–65 (see Appendix A for interview questions). Each interview lasted approximately 15 minutes. The sample size for the qualitative phase was guided by the principle of thematic saturation, whereby data collection continues until no substantially new themes emerge. Given the relatively focused research aim and the convergence of responses across participants, the final sample of 10 inter-

views was considered sufficient to achieve depth and thematic coverage.

The participants in the qualitative phase were recruited as a separate sample using a purposive sampling strategy. The qualitative and quantitative phases were conducted with independent samples. The qualitative phase aimed to provide a deeper understanding of participants' perceptions and to complement the quantitative findings by exploring underlying experiences and meanings.

Quantitative data were analyzed using SPSS. Descriptive statistics were computed for sociodemographic variables, and relationships among variables were examined through Pearson correlation analyses. Qualitative data were analyzed using MAXQDA, following a thematic analysis approach. The analysis process involved initial coding of the data, followed by the identification and refinement of themes based on recurring patterns. To ensure consistency and reliability, two researchers independently coded the data and compared their coding. Any discrepancies were discussed until a consensus was reached.

■ Results and Discussion

Descriptive Analysis:

The majority of the participants were women (68.8%, $n = 218$), and the mean age was 41.52 years ($SD = 12.30$). Most participants held an undergraduate degree (62.8%), followed by those with a graduate degree (30.9%). The mean perceived socioeconomic status was 6.04 on a 1–10 scale ($SD = 1.40$). Additionally, 53% of the participants had previously received support from a therapist, whereas 47% had not. When asked whether they would prefer an AI therapist, 42.0% responded “unsure,” followed by 28.8% indicating “yes” and 19.2% indicating “no.” Finally, regarding data security, 56.8% of participants reported that they would trust a human therapist over an AI system (see Table 1).

Table 1: Sociodemographic characteristics of participants.

The sample was predominantly female and highly educated, and nearly half had previous therapy experience. A substantial portion of participants remained unsure about whether they would prefer an AI-based therapist, reflecting the general ambivalence observed in the study.

	n/M	%/SD
Gender		
Women	218	68.8
Men	99	31.2
Education Level		
Middle School	1	.3
High School	19	6.0
Undergraduate	199	62.8
Graduate	98	30.9
Previous Therapy Experience		
Yes	168	53.0
No	149	47.0
Previous Use of AI Tools		
Yes	281	88.6
No	36	11.4
AI Therapist Preference		
Yes	123	28.8
No	61	19.2
Unsure	133	42.0
Data Security Preference		
Human Therapist	180	56.8
AI Therapist	21	6.6
None	116	36.6
Age (18-65)	41.52	12.296
Socioeconomic State (1-10)	6.04	1.400

Correlational Analysis:

Bivariate correlation analyses were conducted to examine the relationships among the variables (see Table 2). The

results indicated significant associations between attitudes toward AI-based psychotherapy and several dimensions of AI anxiety. Specifically, attitudes toward AI-based psychotherapy were negatively and weakly associated with learning anxiety ($r = -.115, p < .05$). In addition, a negative and significant association was observed between AI configuration anxiety and attitudes toward AI-based psychotherapy ($r = -.163, p < .01$). However, attitudes toward AI-based psychotherapy were not significantly related to job-replacement anxiety or sociotechnical blindness ($p > .05$). Furthermore, attitudes toward AI-based psychotherapy were moderately and positively associated with general attitudes toward AI ($r = .500, p < .01$).

General attitudes toward AI were also significantly related to all dimensions of AI anxiety. Job-replacement anxiety was negatively and weakly associated with general AI attitudes ($r = -.181, p < .01$). Learning anxiety showed a negative and moderate association with general AI attitudes ($r = -.375, p < .01$). Sociotechnical blindness was negatively and weakly associated with general AI attitudes ($r = -.188, p < .01$). Finally, AI configuration anxiety demonstrated a negative and moderate association with general AI attitudes ($r = -.330, p < .01$).

Table 2: Bivariate correlation of the main variables.
AI-therapy attitude showed negative associations with learning anxiety and AI configuration anxiety, and a positive association with general AI attitude. General AI attitude demonstrated consistent negative correlations with all AI-anxiety subdimensions, indicating that anxiety plays a central role in shaping AI-related perceptions.

	M	SD	1	2	3	4	5	6
1. AI-Therapy Attitude	2.75	.82	1	-.036	-.115*	-.083	-.163**	.500**
2. Job Replacement	4.34	1.58		1	.447**	.768**	.687**	-.181**
3. Learning	2.63	1.28			1	.405**	.440**	-.376**
4. Sociotechnical blindness	4.74	1.55				1	.748**	-.188**
5. AI Configuration	4.19	1.88					1	-.330**
6. AIAS-4	6.86	2.06						1

*AIAS-4: AI attitude scale

Qualitative Findings:

THEME 1: Positive Aspects of AI-Based Psychotherapy:

The first theme emerging from the thematic analysis highlights the perceived positive aspects of AI-based psychotherapy. This theme comprises two categories: (1) Technical and practical benefits, and (2) Therapeutic and perceptual benefits.

Category 1) Technical and Practical Benefits of AI Therapy:

Several participants emphasized that AI-based therapists possess extensive, data-driven knowledge structures, which they viewed as a major advantage. According to these participants, AI systems can analyze information drawn from numerous scientific sources, allowing them to operate with a broader and more up-to-date knowledge base. This capability was perceived as enabling more systematic and accurate identification of problems. One participant noted:

“We all graduate from a school, and we can only develop ourselves to a certain extent. But AI filters data from so many articles and sources that it may identify some things more clearly.” (Participant 3, Position 26)

Another participant similarly highlighted the computational benefits of AI:

“It may reduce human error because it works systematically. An AI system processes large datasets. A human can only see a limited number of clients and receive a limited amount of training in their lifetime. AI can analyze vast amounts of data simultaneously and detect better patterns.” (Participant 5, Position 24)

Participants also emphasized cost as a significant advantage. One participant stated:

“Cost is very important. Psychologists have become very expensive recently, while we use ChatGPT for free.” (Participant 10, Position 24)

Thus, rising therapy costs were perceived as limiting access, whereas AI-based services—being low-cost or free—were viewed as increasing accessibility for broader populations.

Another frequently mentioned advantage concerned time efficiency and ease of access. Participants noted that scheduling appointments and adhering to specific time slots can make therapy difficult to access. In contrast, AI tools offer immediate support:

“When you feel upset at that moment, you can ask something without making an appointment.” (Participant 1, Position 72)

Another participant described the potential shift in accessibility dynamics:

“I think human therapists may become a kind of luxury. Therapy is not something easily accessible. As with everything, there will be a widely accessible version and a hard-to-access version. This may affect the value of human therapists and make access more difficult.” (Participant 5, Position 26)

Category 2) Therapeutic and Perceptual Benefits of AI-Based Therapy:

Participants also emphasized several therapeutic and perceptual advantages of AI-based psychotherapy. One commonly mentioned benefit concerned reduced fear of judgment. Some individuals reported that societal stigma surrounding psychotherapy may prevent people from seeking help; therefore, consulting an AI-based therapist could feel safer. As one participant explained:

“If someone does not want to tell others they are going to therapy, or if they do not trust a third person enough to share these things, the barriers become significant.” (Participant 6, Position 25)

Another perceived benefit involved the absence of emotional reactions in AI systems. Participants suggested that human therapists might unintentionally bring their own emotions into the session, whereas AI-based tools are free from such influences. One participant stated:

“Therapists are not supposed to bring their own emotions into the process. Since AI is a machine, it is automatically free of emotions.” (Participant 11, Position 11)

Participants also highlighted that AI systems may approach clients with less bias compared to human therapists. They noted that human therapists, like all individuals, can be influenced by their personal beliefs, experiences, or social norms, which may shape their interpretations or responses—even unintentionally. One participant expressed this:

"People are always biased, even the least biased ones. If you see someone with tattoos, you might not think they are polite before speaking to them. ChatGPT doesn't have that bias. Even therapists can be influenced by such judgments." (Participant 7, Position 23)

Similarly, another participant described the issue in terms of objectivity:

"Every therapist has a different style, worldview, and way of perceiving things, and these vary depending on their training. Even if they try not to, they can still impose their own perspective. Everyone interprets life through their own lens. But with AI, this part could be prevented if the technology is well designed." (Participant 8, Position 25)

Overall, participants perceived AI-based therapy as potentially more neutral and less judgmental, offering a therapeutic space less influenced by human bias or emotional interference.

THEME 2: Negative Aspects of AI-Based Psychotherapy:

Based on the thematic analysis, the second theme identified was the negative aspects of AI-based psychotherapy. Similar to the first theme, two distinct categories emerged under this theme: Category 1 focused on ethical and security-related disadvantages, while Category 2 highlighted concerns related to the therapeutic process and emotional experience.

Category 1) Ethical, Privacy, and Trust-Related Concerns in AI Therapy:

Participants frequently expressed concerns about data privacy in the context of AI-based psychotherapy. Many reported apprehension about the storage of personal and emotional information in digital environments or the possibility that such information might be shared with third parties. One participant stated:

"When I go to a human therapist, I know they will keep my information confidential, but when I give it to an AI system, there may be a data leak. It doesn't give me that sense of security." (Participant 3, Position 24)

These concerns became especially salient when discussing highly sensitive personal matters. As another participant explained:

"Our data are leaked and stolen so often nowadays. So I wouldn't want to share very personal details; I would keep it more superficial." (Participant 4, Position 22)

A different participant described how a negative prior experience shaped her perceptions of AI-based systems:

"Once, while sharing a text, I accidentally mentioned my daughter's name. Now whenever I ask something, she responds by using her name. I really regret saying it; I wish I could make it forget, but it doesn't. It makes me very uncomfortable because it even notifies you that your data has been updated when you share personal information." (Participant 1, Position 56)

While most participants expressed strong concerns about data security, a minority reported that they were not particularly worried about privacy issues. These individuals felt that the vast amount of data processed by AI systems diluted the personal risk:

"I don't worry. I think millions of people use this system—my data is just a drop in the ocean. I actually share personal things, but I don't have security concerns. If there were a leak, I'm sure my data wouldn't be the one anyone cared about. But if I were a famous person, I might have such concerns." (Participant 7, Position 19)

Another recurring issue involved doubts about the reliability and accuracy of information provided by AI systems. Participants questioned whether AI-generated responses could be trusted, especially when compared to those of trained therapists. One participant explained:

"Even psychotherapists who receive extensive training often need years of experience to become truly effective. AI often gives incorrect information. I've seen this many times in my own experience." (Participant 1, Position 66)

Category 2) Therapeutic and Perceptual Disadvantages of AI Therapy:

The primary disadvantage emphasized by participants in this category was the lack of human interaction and relational connection. Human therapists were perceived as advantageous due to their ability to engage in face-to-face interaction and demonstrate emotional understanding. Several participants noted that, through their training, human therapists can interpret nonverbal cues—such as tone of voice, facial expressions, and body language—and can even sense emotions that clients struggle to articulate. Participants stated that AI systems currently lack this level of nuance. As one participant explained:

"I don't think it would understand, because the human mind is very complex. Even if people seem similar, they differ in very subtle ways, and these subtle things carry great meaning. Sometimes, even if you cannot fully articulate it, you can feel it based on past experiences. An AI system cannot have that sensitivity." (Participant 5, Position 18)

Another disadvantage frequently mentioned was the absence of in-person interaction itself. Participants emphasized the value of being physically present with another human being in the therapy room, suggesting that such interaction contributes to a stronger therapeutic alliance. One participant stated:

"Psychology is one of those fields where you really need human-to-human interaction—conversation, flow, and presence—so that the person can feel understood. Looking into someone's eyes, seeing their face, seeing someone like yourself... that makes people feel better. That's why human psychotherapy is very important." (Participant 8, Position 28)

Several participants also pointed to the lack of authenticity and realism in AI-based interactions. They felt that AI systems tend to avoid confrontation and may respond indirectly even when a user expresses clearly incorrect statements. As one participant described:

"Even if it sees something completely wrong, I don't think it would say, 'This is wrong.' It's not in a mode to challenge people. For example, Grok used to argue with users, but ChatGPT and similar systems are not at a level where they insist that something is wrong—they keep trying to soften or redirect the conversation. So it doesn't behave like an expert." (Participant 7, Position 21)

Another participant reflected on the idea that, although AI may not truly understand emotions, it can still create a feeling of being understood:

"It's debatable what 'understanding' really means, but it can make the other person feel understood. We all need to feel understood, and it doesn't need to give major solutions—just framing and reflective questions like 'I understand you' or 'You seem worn out.' Even if it doesn't actually understand, I would still feel understood, so in that sense, yes, it understands." (Participant 6, Position 16)

■ Discussion

This study aimed to examine individuals' perceptions of AI-based psychotherapy using a mixed-methods approach. In the quantitative phase, descriptive findings indicated that the mean age of the sample ($M = 41.52$, $SD = 12.00$) reflected a wide age range; however, the relatively higher average age may have contributed to more cautious attitudes toward AI. Supporting this interpretation, Kubovics¹⁶ found that age is a significant predictor of attitudes toward AI, with older adults reporting more negative views and lower willingness to share data with AI systems. Nearly half of the participants had prior therapy experience, which likely enabled them to compare AI-supported interventions with human-delivered psychotherapy. Conversely, participants without therapy experience may have responded more speculatively.

Correlation analyses demonstrated meaningful associations between attitudes toward AI-based therapy and several psychological and cognitive factors. Learning anxiety was negatively associated with attitudes toward AI-based therapy, indicating that higher levels of learning-related anxiety correspond to lower willingness to engage with AI-supported psychotherapy. This finding highlights the importance of user-friendly interfaces and simplified system design to reduce user-related concerns (see also Kleine¹⁷). Similarly, AI configuration anxiety was also negatively associated with attitudes toward AI-based therapy, suggesting that concerns related to the technical functioning, transparency, and reliability of AI systems may undermine acceptance of AI-supported interventions. This interpretation is consistent with qualitative findings highlighting fears related to data security, privacy breaches, and the possibility of inaccurate information.

Moreover, general attitudes toward AI were positively associated with attitudes toward AI-based psychotherapy. Individuals who held favorable views of AI in general also evaluated AI-supported therapy more positively. Practically, this suggests that broader efforts to improve public understanding and acceptance of AI technologies may indirectly enhance openness toward AI-based mental health interventions.

Importantly, the qualitative findings help contextualize these quantitative associations by elucidating the underlying reasons behind participants' attitudes. Specifically, the themes derived from the interviews indicate that participants' ambivalence and trust-related concerns are primarily driven by perceived limitations in emotional authenticity, data security, and the absence of human interaction, thereby providing a deeper explanation

for the observed relationships between AI anxiety and attitudes toward AI-based psychotherapy.

Building on this integration, the qualitative findings further illustrate how these concerns are reflected in participants' concrete evaluations of AI-based psychotherapy. These findings indicate that participants primarily emphasized technical and practical advantages of AI—such as cost-effectiveness, time efficiency, accessibility, and large-scale data processing—while describing emotional and relational limitations as key disadvantages. Although AI-based therapy was perceived as convenient and functionally helpful, participants expressed reservations about forming an emotional connection or fully “internalizing” the therapeutic process. On the therapeutic side, the absence of emotional reactions, reduced fear of judgment, and freedom from human bias were perceived as potential benefits. Conversely, concerns regarding data privacy, doubts about the accuracy of information provided by AI, and risks of misinformation were prominent barriers. Participants also frequently noted the lack of face-to-face interaction, emotional depth, and authenticity in AI-based therapy, indicating that such systems cannot yet replicate the interpersonal and relational qualities of human psychotherapy.

Similarly, most participants had previous experience using AI tools, suggesting that attitudes toward AI are shaped by prior exposure. Regarding preferences for AI-based therapy, most participants reported uncertainty; still, 28% indicated they would consider using an AI therapist, whereas 19% stated they would not—an overall pattern consistent with the dual structure observed in the qualitative findings. This ambivalence can be understood as stemming less from a lack of familiarity with AI and more from concerns related to the nature of the therapeutic process itself. Given that the majority of participants reported prior experience with AI tools, the uncertainty is unlikely to reflect low AI literacy. Rather, the qualitative findings suggest that participants' hesitation is rooted in issues such as data security, limited emotional authenticity, and the absence of face-to-face interaction. These factors indicate that participants may recognize the practical advantages of AI-based psychotherapy while simultaneously questioning its ability to meet relational and emotional needs. Thus, the “unsure” responses may reflect a tension between perceived utility and perceived limitations, rather than simple unfamiliarity with the technology. Previous research by Aktan *et al.*¹⁷ reported that 50% of participants preferred an AI therapist; however, their study did not include an “unsure” response option, which may partly explain the discrepancy. In terms of data security, more than half of the participants trusted human therapists, while only 6% trusted AI systems—again aligning with our qualitative results. Overall, both quantitative and qualitative data suggest limited trust in AI-based psychotherapy, though not a complete absence of trust.

An additional noteworthy finding is the discrepancy between participants' willingness to consider AI-based psychotherapy and their low level of trust in AI systems regarding data security. This “trust gap” suggests that individuals may be willing to use AI-based services despite reservations about privacy and security. One possible explanation is the distinction

between perceived utility and trust: participants may recognize the practical benefits of AI—such as accessibility, cost-effectiveness, and immediacy—while simultaneously maintaining concerns about how their data are handled. In this sense, usage intentions may be driven by convenience and situational needs rather than full confidence in the system.

Furthermore, qualitative findings indicate that participants may regulate their use of AI by limiting the depth or sensitivity of the information they share, thereby managing perceived risks. This selective engagement allows individuals to benefit from AI-based support while minimizing potential privacy concerns. Thus, the observed trust gap does not necessarily reflect a contradiction, but rather a pragmatic adaptation to the perceived trade-offs between utility and risk.

These findings should also be interpreted in light of the sample characteristics. The predominantly female and highly educated composition of the sample may have influenced perceptions of both data security and AI-related risks. Prior research suggests that women tend to report higher levels of AI-related anxiety and greater sensitivity to potential risks, particularly in contexts involving personal data. Similarly, individuals with higher education levels may be more aware of issues related to data privacy, algorithmic bias, and technological limitations, which could lead to more cautious or critical evaluations of AI-based psychotherapy. In contrast, more diverse or less technologically informed populations may exhibit different patterns of trust and concern, potentially placing greater emphasis on accessibility and convenience over perceived risks. Therefore, the observed levels of uncertainty and limited trust in AI systems should be interpreted within this demographic context.

■ Limitation

This study has several limitations. First, the sample consisted predominantly of women. Prior research indicates that women tend to report higher levels of AI-related anxiety and hold more negative attitudes toward AI compared to men (Russo *et al.*¹⁸; Sindermann *et al.*¹⁹). Therefore, our findings may reflect a female-dominant pattern of attitudes. The gender imbalance in the sample also limits the generalizability of AI-related attitudes. Additionally, most participants had undergraduate or graduate education and perceived their socioeconomic status to be above average, which may confer an advantage in terms of AI literacy and understanding of technological concepts (e.g., Sebetci²⁰). Hence, our findings cannot be generalized to individuals with lower socioeconomic and educational backgrounds. Another limitation concerns the potential cultural specificity of AI-related attitudes. As all participants in our study were Turkish, the findings may be influenced by cultural factors that shape perceptions and expectations of AI-based therapies, thereby restricting generalizability (Yam *et al.*²¹). Finally, the correlational and cross-sectional nature of the study limits the extent to which causal inferences and broader generalizations can be made. Future studies should include broader age groups, clinical populations, and individuals with no prior experience with AI to enhance the generalizability of the find-

ings. Moreover, experimental and longitudinal designs would provide valuable insight into the long-term effectiveness of AI-based therapies and the processes through which users adapt to such systems.

■ Conclusion

This study provides a comprehensive contribution to the limited body of literature by examining societal perceptions of AI-based psychotherapy through both quantitative and qualitative data. The findings indicate that levels of AI-related anxiety and general attitudes toward AI are closely linked to attitudes toward AI-supported psychotherapy. Additionally, although participants acknowledged the practical advantages offered by AI, they emphasized that AI systems cannot fully replace human therapists in the emotional and relational dimensions of the therapeutic process.

If AI is to be integrated as a supportive tool within psychotherapy services, it will be essential to design user-friendly interfaces, enhance transparency regarding data security, and strengthen AI's capacity for emotional closeness or empathic communication. Furthermore, developing hybrid human-AI therapeutic models may allow individuals to benefit from the practical conveniences of AI while also meeting their need for a meaningful therapeutic relationship.

From a practical perspective, the findings of this study carry important implications for multiple stakeholders. For mental health practitioners, the results highlight the continued importance of the human element in therapeutic processes, suggesting that AI should be integrated as a supportive rather than a substitutive tool. For developers of AI-based mental health applications, the findings emphasize the need to enhance transparency, data security, and the capacity for emotionally responsive interactions. Addressing users' concerns regarding privacy and emotional authenticity may increase trust and acceptance of such systems. Finally, for policymakers and institutions, the results underscore the importance of establishing ethical guidelines and regulatory frameworks to ensure the safe and responsible use of AI in mental health services.

In sum, this study highlights both the potential and the limitations of AI-based psychotherapy and offers important insights into the future of digital mental health services.

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The Role of Artificial Intelligence in Crop Health Monitoring: Current Applications and Future Prospects

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ABSTRACT: This paper analyzes how artificial intelligence is transforming current-day agriculture with improved monitoring, prediction, and decision-making systems. It discusses how models of machine learning, remote sensing, and data-driven solutions are making it possible to have more efficient crop management, detect stressors earlier, and more accurately use water and other resources. The review examines the main uses of AI in precision agriculture, namely, disease detection, yield forecasting, soil analysis, and nutrient analysis, as well as climate adaptation strategies. The most important technological solutions, including convolutional neural networks, multispectral imaging, and sensor-integrated platforms, are studied to reveal their potential and real-life constraints. The paper also takes into account the issues that hinder the massive adoption of AI for crop health monitoring solutions. Furthermore, the discussion provides future development opportunities, including the role of better datasets, hybrid methods, and low-cost field-ready solutions in increasing AI contribution to global agriculture. In general, the article contends that although AI will not be able to take over traditional farming knowledge completely, it can contribute a lot to agricultural systems in the aspect of decision-making and resilience when used in a responsible manner.

KEYWORDS: Machine Learning, Agriculture and Agronomy, Precision Agriculture, Crop Health Monitoring, Artificial Intelligence.

■ Introduction

Farming uses nearly 70% of the world's freshwater supply, and a significant portion of this water is wasted because of inefficient irrigation methods and outdated farming practices.¹ The challenges that farmers across the world have to face in their work continue to make it difficult to derive stable crop outputs due to persistent issues with pest and disease detection, inefficient resource usage, labor shortages, inaccurate yield predictions, and climate changes that are difficult to predict. Conventional approaches to farming, including visual checking of crops, fixed watering systems, and estimation of yield in accordance with historic averages, are not always effective in optimizing agriculture. Traditional methods of crop health monitoring make it difficult to identify threats in time, misuse resources, and are slow to react to environmental pressure.²

The emergence of artificial intelligence (AI) as a new opportunity at the end of the 2010s has provided the answer to this long-standing question: Can smart algorithms and sensor-driven systems assist farmers in overcoming these challenges to make agriculture more precise, efficient, and resilient? AI is also efficient at handling large amounts of data, such as satellites, drones, and ground sensors, and finding the patterns that might possibly have passed unnoticed by a human. As an example, AI-powered drones would be able to survey fields and detect the initial signs of crop stress, pest outbreaks, and nutrient deficiencies- then it would become possible to do something before significant harm is caused.³ Similarly, real-time irrigation optimization by sensor-based system integration of soil-moisture data sets, weather forecasting, and crop models is capable of reducing the wastage by a signif-

icant factor, but preserving and/or enhancing the health of the plants.⁴ Robotic systems are also alleviating labor shortages, an issue of growing concern in most agricultural areas, by allowing weed and pest elimination with extreme accuracy, eliminating human-intensive fieldwork, including advanced drone prototypes.⁵ In the meantime, AI-trained yield-forecasting models combine past data with real-time environmental data to increase the prediction accuracy by as much as 30 percent compared to traditional approaches. And lastly, as climate variability emerges as the new reality, AI solutions are helping to ensure climate resilience, simulating the response of crops to changing conditions and advising on how to plant or manage crops in the future.⁶ As Gupta *et al.* state, "[AI] supports farmers in adapting to climate change by analyzing vast amounts of environmental data to predict weather patterns, droughts, and other climate shifts."¹⁰ AI is becoming an increasingly valuable asset to farmers.

Though there are several review articles that explore the potential of AI in precision agriculture, the majority of them concentrate on a single area, i.e., optimization of irrigation or monitoring with drones. The current review is unique in its systematic coverage of the five interrelated challenge areas, including the late pest/disease detection, inefficient resource use, labor shortages, yield-prediction errors, and climate adaptation, all in the prism of central coverage of crop health monitoring.

■ Methodology

This paper examines the implementation of artificial intelligence in precision agriculture to solve problems, including the identification of pests and diseases, managing resources,

predicting yields, and adapting to climate change. In order to have a balanced and comprehensive view, academic databases such as Google Scholar, ScienceDirect, PubMed Central, and ResearchGate, and reputable industry publishers, such as Nature and MDPI, were used to collect research articles. The search terms involved the use of the keywords: "AI in precision agriculture," "AI crop yield prediction," "crop monitoring with drones," "AI pest and disease detection in agriculture," and "AI climate resilience in agriculture."

Inclusion criteria were that the studies must be published between 2015 and 2025 with a specific interest in AI-based agricultural applications, typically with a quantifiable outcome, i.e., accuracy data, efficiency gains, or verified cases of application in real agricultural settings. The studies that talked about generic automation without AI elements or those dated earlier than 2015 were usually not considered to guarantee relevance to contemporary technological developments.

The papers were filtered based on their technical aspects (e.g., machine learning models, computer vision, IoT-enabled sensing) and the application in agriculture (e.g., optimizing irrigation, detecting crop health, predicting yields). Results were then divided into five broad problem areas, which are late pest and disease detection, inefficient utilization of resources, labor shortage, inadequate yield forecasting, and climate change resilience. This methodological structure facilitated the direct comparison between the AI methods and their practical effects.

Lastly, the focus on multiple research works and the narrowing down of literature to AI-based studies with measurable outcomes creates a coherent and precise image of how AI is changing the methods of health management and farm operations of crops. The given methodology also preconditions the discussion of the important findings and their implications in terms of practice, policy, and further research.

■ Discussion

Precision Agriculture and Crop Health Surveillance:

Precision agriculture (PA) is one of the revolutionary moves in contemporary farming that involves a more data-driven and antipassive approach to decision-making by using state-of-the-art sensing, imaging, and AI-based analytics.^{7,8} Although the traditional system is mostly based on human control and regular routines, PA combines satellite shots, drone technology, and IoT devices to offer twenty-four-hour and high-resolution data on crops. This enables stress, disease, or pest presence to be detected prior to it becoming visible or causing economic harm.^{9,10}

In a carefully controlled study, it was shown that deep convolutional neural networks could identify 26 diseases in 14 crop species with almost perfect accuracy.⁹ Findings of the authors point to the possibility of AI being faster and more accurate than human beings. Nevertheless, they also note a severe drawback: in the case of models being presented to uncontrolled conditions in the field, performance can decrease to a significant degree. This dissimilarity represents one of the research gaps, which is the ability to translate laboratory-level accuracy to real-life, heterogeneous settings, and remains a critical issue.

It also highlights the need to have strong and adaptable models, which are critical to their deployment in various climates and geographies.

The combination of multi-source datasets allows machine learning models to make optimal decisions on irrigation, fertilization, and pest control. Practically, it implies that the farm managers will be able to introduce site-specific management approaches, which may lead to decreased levels of both unnecessary application of chemicals and water wastage. However, a combination of several data streams (e.g., weather, soil, crop stage, etc.) can be challenging to calculate and logistically difficult, especially in developing countries, where fast internet and sensor networks are not widely available.^{4,7,11}

In addition, the implementation of decision support systems (DSS) into PA enables farmers to make uncertain decisions proactively. DSS platforms are not only capable of simulating various management situations but also offer probabilistic results that are better at risk assessment and planning. This represents a major change in agronomic thinking: farmers can predict their problems in advance, in contrast to having to respond to problems that can be observed. This is extended by robotics and autonomous field platforms that can conduct routine monitoring at high spatial and temporal resolution without requiring human labor and allowing greater scalability.⁴

More importantly, the adoption gap is still broad, although the evidence is positive concerning the promise of PA. Most of the studies are limited to either pilot-scale implementations or specific crop species,^{7,9} which does not allow us to generalize. It is also stated in the literature that the interdisciplinary nature of PA demands coordination: agronomists, data scientists, engineers, and policymakers need to be coordinated to achieve success.¹⁰ The lack of consideration of this may hinder adoption even where technological potential is illustrated.

To conclude, PA is not only a tool but a change in crop management paradigm. It allows conducting interventions earlier, to use resources more efficiently, and to obtain higher yields because it changes the reactive to predictive practices. However, the most important thing is to reduce the gap between the experiment performance and the actual field operational reliability to make sure that these systems are flexible, understandable, and available to different farms and areas. The evidence indicates that although PA is transformative, to reach the consistent benefits, attention must be paid to technological, environmental, and social aspects.

Traditional Methods: Limitations and the Need for AI:

Conventional methods of crop control rely heavily on human labor, inspection, and predetermined irrigation and fertilization timetables.^{7,8} The approaches are reactive in nature and can only identify stress or disease when the symptoms are visible. They are efficient in small and homogeneous plots, but they become inefficient in large or diverse fields, where crops are subject to unnoticed pressure or pests.^{7,10}

Human examinations are unclear and unstable. Even experienced scouts may fail to detect the early symptoms of a disease or even fail to notice the minor nutrient deficiency, depending

on the conditions of the environment. The FAO measures the effect of reactive management, stating that one of the primary reasons for crop loss and resource wastage in the world is the misallocation of water and fertilizer. These are exacerbated by labor shortages, the typical occurrence during peak seasons of farming activities in most areas.¹³

The solution to these problems is provided by AI and machine learning (ML) technologies through the data solution. With sensors, drones, and satellite images, AI systems will be able to identify even the slightest shifts in the health of crops well in advance of their appearance.^{7,9} It was demonstrated that AI is able to give accurate recommendations on irrigation, fertilization, and pest management to take timely and location-specific measures. This minimizes resource wastage and enhances the resistance to unpredictable environmental factors, making it a great shift towards reactive to predictive crop management.¹⁰

Nevertheless, there are critical limitations and adoption barriers that are identified in the literature as well. A large number of AI projects in the experimental stage are specific to particular crops, making them less generalizable to other regions or types of crops.^{7,9} Smaller farms usually have difficulties accessing the required IoT network, drone technology, or high-speed internet, which prevents real-world use.^x Moreover, many AI algorithms are of a black-box nature, which can diminish user trust.¹⁴ Clarifying AI models is indispensable to establishing trust in farmers to make the recommendations provided by the system understandable and practical.¹⁰

In a bigger perspective, these constraints demonstrate that the social and infrastructure obstacles to using AI are no less significant than the technological barrier. The implementation has to be successful by investing in sensors and connectivity, training the farmers and extension agents, and finding effective ways of integrating the AI recommendations into the traditional farming way of operations.^{10,11} More importantly, AI is not to substitute agronomical knowledge but to complement human decision-making by allowing farmers to use their skills in applying it to bigger and more complicated processes.

Overall, traditional practices form the basis of farming, but are unable to support the demands of large-scale, modern, and high-value agriculture. Precision agriculture through AI can be used as an alternative, and it enhances detection, resource use, and predictive control. To achieve these benefits, however, there have to be practical obstacles that are overcome, such as technological, financial, and social barriers. AI should be developed in such a way that it is not a substitute for human knowledge. The evidence shows that the development of AI is a socio-agronomic problem and technological innovation, which must be carefully incorporated into the current systems to achieve maximum benefits and equity.

AI Applications in Key Problem Areas:

Early Pest and Disease Detection:

Precision agriculture requires the identification of pests and diseases early. It has a direct influence on crop yield, and it assists in minimizing the use of chemicals. The conventional scouting mechanisms are based on the use of periodic manual

inspections, which are slow and subjective. They tend to overlook early infestations, particularly in big or diverse fields.^{7,8} Artificial intelligence devices, in particular, computer vision and deep learning, can be used to automate alarming and provide alerts in near real-time. This enables farmers to act before outbreaks deteriorate.

Researchers applied a deep convolutional neural network to the PlantVillage dataset in a controlled environment study (Figure 1). It had a 99.35 percent accuracy in the controlled environment when identifying 26 crop diseases in 14 species. This demonstrates how AI is able to outcompete human scouts regarding speed and precision. Nevertheless, the experiment also revealed an alluring decline in accuracy (to approximately 31 percent) when it was directed to images that were taken in uncontrolled environments, including images from trusted on-line sources and academic agriculture extension services. This refers to the difficulties of extrapolating AI models. In any real-life scenario, AI should be able to support environment, lighting, and crop development cycle variations to be effective in critical scenarios.⁹

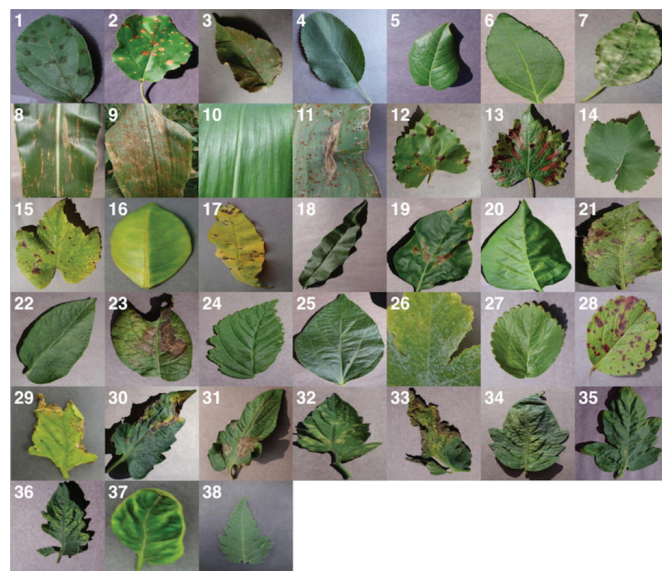


Figure 1: Leaf Samples from the PlantVillage Dataset. This figure illustrates the type and visual diversity of input data used to train convolutional neural networks for automated disease classification, highlighting both the potential and the generalization challenges when models trained on controlled images are applied to field conditions. Adapted from: <https://www.frontiersin.org/journals/plant-science/articles/10.3389/fpls.2016.01419/full>

Drone imaging, when paired with AI, can support the high-resolution regulation of extensive fields quite well. The use of multispectral imaging on drones and processing with the convolutional or recurring neural networks can detect the slightly obvious signs of pest or disease infestations that can be very small and can be impossible to notice.¹⁰ Drones could also help alleviate the problem with localized pest management. This will help in the minimization of pesticide overuse. Alazzai supports this and demonstrates that by integrating the UAV data with ground sensor networks, predictive analytics can be implemented.¹³

Although these systems are promising, they come with barriers to their implementation. Annotated datasets, which are

high-quality, are essential to the development of AI models, yet such datasets are frequently unavailable for some crops, diseases, or environmental factors.^{7,9} Also, smallholder farmers do not necessarily have access to drones, IoT sensors, or computer resources to implement them.^{4,11} Therefore, although AI has serious theoretical and experimental advantages, the practical implementation must concentrate on infrastructure, training, and model flexibility regarding early pest detection.

There is an indication that AI may transform the way the early detection of pests and diseases is done, yet the rate of adoption will be uneven. AI can help in curbing the losses of crops and chemical costs in high-value or large-scale farming, where infrastructure and funding are available. In smallholder settings, AI must be combined with readily available hardware, low-priced sensors, and a straightforward interface to offer fair and practical gains. It is also required that research be conducted on transferable models that will be accurate in other regions, crops, and environments.

Inefficient Resource Usage:

Using water, nutrients, and other farm inputs efficiently remains a major problem in conventional agriculture. Old-fashioned ways of irrigation and fertilization are usually worked out according to timings or the intuition of the farmer. It may result in under- and over-application of needed resources for plants.² The excessive use of fertilizers and water reduces the economic performance and damages the environment by reducing soil nutrients, contaminating water, and contributing to greenhouse gases.

Precision agriculture through AI provides resourceful data management. AI-enabled irrigation systems that utilize soil moisture sensors, weather forecasts, and crop growth models can reduce water consumption by a factor of up to 30 percent without affecting yields.⁸ On the same note, machine learning models have the ability to adjust the application of fertilizers to the targeted sites by estimating the amount of certain nutrients each site requires to maintain a healthy crop without causing overuse.⁷

By combining IoT sensors and predictive analytics, it is possible to monitor soil nutrients, moisture, and plant health indicators in real time.^{4,13} To illustrate, AI algorithms will be able to turn on irrigation only when the moisture in the soil decreases to a certain amount, depending on the crop, or recommend the use of nitrogen only after considering realistic plant requirements and not previous average levels. These recommendations can be performed precisely by robotics and automated systems and lead to additional waste reduction.¹²

Although the results seem to have definite advantages, there are challenges in the practical implementation. The cost of sensors, IoT networks, drones, and automated systems may not be easy to meet at the beginning, particularly in the case of smallholder farms.^{4,11} Also, a large number of AI models are trained with pilot-scale or high-resolution datasets, which might not be relevant to new types of soil, crop, and climatic conditions. This poses some important questions regarding the dependability and extensibility of the resource optimization models to diverse farming systems.

The human aspect of adoption is another important factor. Farmers need to believe in AI advice and understand how to respond to it, even with the technology in place. Explainable AI systems are essential to converting predictive results into practical choices that are consistent with the local farming experience and conditions.¹⁰ This may otherwise be achieved, meaning that failure to do so may lead to under-utilization or mis-utilization and hence cause restriction in the efficiency gains.

Finally, AI can significantly enhance efficacy in resource use, which is not only cost-effective but also environmentally friendly. Nonetheless, fair deployment must put into focus infrastructures, expenses, model universality, and confidence among users. It is crucial to bridge the research-practice gap, and future research must investigate cost-efficient, scalable, and comprehensible AI applications in various agricultural contexts.

Labor Shortages:

Labor shortage is a regular problem in agriculture, particularly during the important times such as planting and harvest. Operations such as scouting, weeding, and harvesting that were previously done by manual labor are not as sustainable as they used to be. This is especially accurate in regions where there is migration to cities, an aging agricultural sector, or seasonal labor shortages.^{7,13} This deficiency limits the productivity of farms and postpones the timely response to pests, diseases, and a lack of nutrients, which directly affect the outputs and profits.^{8,15}

Robotic systems powered by AI offer a potential solution. Drones, weeding robots, and monitoring systems can effectively perform repetitive or labor-intensive duties by autonomous harvesters. Robotic strawberry harvesting machines are equally accurate at their work as human workers, indicating the possibilities of robotics in sensitive farming jobs.¹⁶ In addition to harvesting, autonomous robots equipped with imaging sensors can be used to carry out constant measurements, eliminating human field inspection, and allowing crop stress to be identified earlier.^{10,12}

In spite of these developments, there are huge challenges to the adoption of these technologies. Small and medium farms cannot afford the high initial costs and continual maintenance costs.^{4,11} Moreover, AI-powered robotics may need special skills to operate and maintain, and farmers with no access to training or support find it difficult to operate them. There are also crop-specific restrictions: not all crops can be effectively harvested and controlled using robots at the moment because of structural complexities or sensitivities.

In a broader view, AI and robotics may change the place of work in farming. They are able to make human work less monotonous, more supervisory, decision-making, and analytical. The change has the potential to improve the efficiency of farms, reduce labor shortages, and provide more opportunities to respond to the stress of crops or problems with pests in time.^{7,13} Nonetheless, the implementation of robotics should not ignore economic and social realities to guarantee that the benefits are reaped without increasing the disparities between

large farms run by industrial enterprises and smaller ones. Further studies are required to develop more affordable yet flexible and user-friendly robotic systems to reduce the gap that currently exists between technology and its practical application in different farming environments.

Yield Prediction Inaccuracies:

Precise forecasting of yields is crucial in the management of farms and also in larger-scale agricultural planning. The conventional forecasting techniques involve the average of the history, farmer surveys, and the use of simple estimates based on previous yields. Nevertheless, these techniques are not always able to reflect the spatial change, temporal change, and real-time environmental dynamics.^{7,8} This may lead to making bad decisions concerning planting, harvesting, and marketing, which may eventually damage profits and food supply stability.

The use of AI-based models can significantly enhance the accuracy of prediction by adding analysis on top of remote sensing, soil maps, climate factors, and crop growth models. Deep neural networks were developed to predict maize yield for the Syngenta Crop Challenge with root mean square errors (RMSE) of about 12% of the average yield (Figure 2).¹⁷ This points to the advantages of AI analysis. The models assist farmers in estimating the level of production, optimizing supply chain planning, and reducing post-harvest losses.

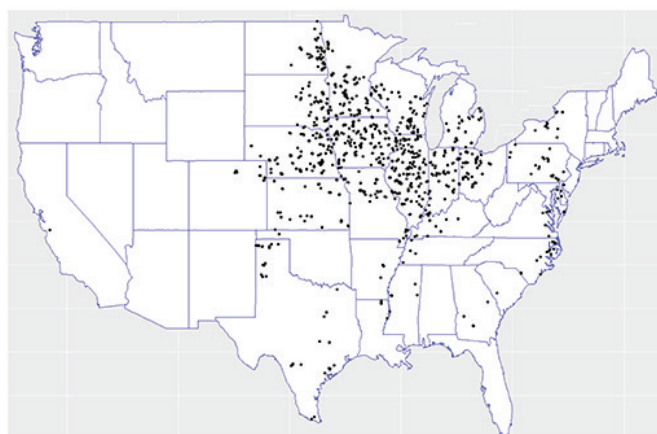


Figure 2: The Maize Hybrid locations across the United States were collected from the 2018 Syngenta Crop Challenge. Adapted from: <https://www.frontiersin.org/journals/plant-science/articles/10.3389/fpls.2019.00621/full>

Integrating various sources of data increases the reliability of a model as well. A combination of satellite imagery and IoT sensor data increases spatial resolution.¹⁰ This is because it provides the opportunity to have field-level specific yield estimates, instead of general regional estimates. This also allows models to capture microclimate and soil variability across the area. This provides a stronger and more adaptive agricultural system that is able to cope with resources and interventions.

Even though conventional yield forecasts take into account the past yield averages, they often fail to capture field-specific variability, which results in inaccuracies in various crops, soils, and climates.^{7,9} The limitations can be overcome through AI-based models by incorporating multi-source data, such as satellite imagery, soil properties, climate conditions, and inputs

from IoT sensors. This combination provides more accurate prediction and makes it possible to forecast dynamically and at the field level.^{4,11}

Despite these improvements, challenges remain. One of the factors that can affect the trust and adoption of AI is that complex AI algorithms like deep neural networks might not be easily interpretable by farmers or policymakers.¹⁰ To achieve the greatest benefits of AI in yield prediction, the models should be generalizable, interpretable, and have a strong data infrastructure. The availability of open-access datasets and explainable AI models is essential to making sure that the yield predictions enabled by AI can help advance the management of resources, mitigate risks, and decision-making in a variety of agricultural environments.^{10,12}

Climate Change Adaptation:

Modern agriculture is challenged by climate-related variability and extreme weather events. The growth in cases of droughts, unpredictable precipitation, stress, and the emergence of new pests complicate the process of yield projections and endanger the producers.^{10,15} Artificial intelligence (AI) and associated data technologies are also being viewed as a necessity, in this case, not only in terms of efficiency but also as the means of farm resilience construction. The literature that already exists constructs the role of AI in adaptation in the context of three main capabilities, namely: (1) the ability to identify risks and issue early warnings at the field level, (2) the ability to provide decision support based on scenarios, which can integrate climate forecasts alongside agronomy, and (3) the ability to provide adaptive operational control (such as irrigation and fertilization) to lower centrality to extreme events, which may be achieved through Geographical Information Systems.^{4,13,15}

Early warning systems and risk detection systems are based on various sensors and pattern recognition. Machine learning models are fed with high-frequency remote sensing (satellites and UAVs) and in-field IoT data (soil moisture and canopy temperature). These models are able to find abnormalities in the health of crops before they become visible.⁷ The recent reviews point out that the combination of these sources of data makes the risk map more accurate, and the producer can understand that this or that particular area is likely to experience drought stress or pest outbreaks according to the future weather patterns.^{10,12} The practical advantage is obvious: the preventive measures could be used where the need is most, and it would help to save water and reduce the losses in yields caused by unpredictable and changing climate.

Scenario simulation is another field where AI is useful because it can be used to simulate various management possibilities against climate forecasts to estimate their possible results. New decision-support systems enable users to experiment with different aspects, including planting dates, planting drought-resistant varieties, or more or less frequent fertilization, and view the potential effects on yield, water use, and profits.^{4,10} It is a major change: instead of only reacting to the emerging issues, farmers are able to rely on evidence-based knowledge to change their operations in advance. These

scenario tools are especially beneficial on the landscape level, where a coordinated response (such as staggered planting among producers, or changing regional water distributions) can reduce systemic risks.

Operational adaptation relates forecasts and situations to automated or semi-automated steps. By incorporating soil moisture sensors, weather data, and simple crop-water models, precision irrigation systems that can apply water only to stressed zones, depending on specific thresholds, can be connected.^{13,19} In case of robotic applicators and variable-rate machinery, the mitigation strategies could be applied with highly accurate control in the following cases, in which AI could apply biocontrols in areas at risk or shade vulnerable plots. This minimizes unwanted inputs in sub-optimal situations.^{12,19}

There are, however, several limitations cautioned in the literature. First, high-quality long-term data, based on which local climate extremes and reactions of crops can be recorded, is required to achieve model reliability; not all regions are covered by such data, making it impossible to calibrate models and adapt them to local conditions.^{11,18} Second, weather prediction is always unpredictable, and its integration with AI adds complexity to the model, which may pose a problem in interpretation and build confidence among the users.⁴ Third, some smallholder farmers might not afford the equipment and connection required to maintain continuous observation and automated responses (such as UAVs, dense sensor networks, edge/cloud computing), which results in the unequal distribution of adaptation benefits.^{15,19} Finally, the social and institutional factors, including the ownership of data, its maintenance capacities, and the extension services, are critical in determining whether predictive signals would be implemented in practice or not.¹⁰

Three priorities are recommended in recent reviews to make it more applicable and fair. The first is localization, which entails coming up with mechanisms that enable models that are trained in dense data to adapt fast to local conditions without the need to have much new data.¹⁸ The second attempt is to make models easy to use and understand through the demonstration of probabilistic forecasting and actionable recommendations with defined confidence levels. This assists farmers in making effective choices without any need for intense expertise.^{10,12} The third priority is designing sensing solutions that are scalable and low-cost, such as low-cost soil probes and smartphone-based imaging, to ensure that technology is accessible to risk producers.^{4,11}

To sum up, AI is an important aspect of climate adaptation, which improves early detection, informative decision-making, and automation of adaptive responses. Nonetheless, to grow resilience fairly, it is necessary to pay attention to the quality of data, its transferability, interpretability, and access to infrastructure in such a way that smallholders and areas with limited resources can benefit, along with more resourced ones.

Critical Evaluation, Contradictions, and Limitations:

Precision agriculture based on artificial intelligence (AI) has a high potential in various aspects, such as disease detection, resource utilization, yield prediction, labor assistance, and cli-

mate-change adaptation. Still, a more careful examination of the literature available shows a complex situation with numerous caveats and unanswered questions.

Generalizability and reliability are the first issues of concern. High-quality datasets and controlled experiments, such as those of Mohanty *et al.*, have high predictive accuracy in disease diagnosis at controlled imaging conditions. However, when these models encounter real-life variations, e.g., lighting variations, heterogeneous crop species, different soil quality, and unsystematic imaging, they tend to reduce the accuracy significantly. In the same vein, Chlingaryan *et al.* and Khaki and Wang state that models of crop yields created based on past data might not work in adverse environmental conditions. This points out the constraints of data-only solutions unless they are locally adjusted or calibrated.

Second, there are major problems with data infrastructure and quality. As a number of studies note, the performance of AI requires large quantities of high-resolution, well-labeled, and timely datasets.^{11,12,18}

As a matter of fact, in most regions, particularly small farms in developing nations, there is neither historical nor current agricultural information, and thus the extent to which such models can be utilized. The presence of noise or data gaps, even with the assistance of sensors, UAVs, and remote sensing tools, may affect the reliability of recommendations, even in cases where the instrument does not operate correctly, requires maintenance, or is connected.^{4,13}

Third, the price and the availability of technology restrict the applicability. Robotics systems, IoT-based sensors, UAV platforms, and cloud analytics are expensive to start and maintain. Although the bigger commercial farms might be able to recoup the invested capital in terms of enhanced efficiency, the small ones may not be in a position to embrace such technologies. This poses a further problem of productivity and resilience inequalities.^{10,15,19} With potentially promising cheap sensors or smartphone imaging, these technologies demand technical skills, maintenance, and regular data input, which may be hard to achieve in resource-strained regions.¹¹

Fourth, the explanation of the functioning of AI models and their development of trust is one of the biggest challenges. A lot of useful AI tools, in particular, deep learning models used to identify diseases or forecast yields, are black boxes.¹⁴ There is not much transparency regarding the decision-making process in these models. AI can also be resisted by farmers who would not implement the models even when technically valid, without proper explanations. It is important to mention that the literature emphasizes the increased attention to explainable AI frameworks that can deliver confidence scores, visual reasoning, or simplified outputs to enhance trust and usability.^{4,18}

Fifth, the environmental and social implications of AI utilization are not clear yet. Efficiency gains, including less water and fertilizer, control of pests in a more targeted way, and less labor, are documented in many studies. Nevertheless, the ecological impact of AI-enhancing agriculture in the long term is largely unexplored.¹⁹ An example is that automated irrigation and fertilization may result in soil depletion if models are short-term optimal, or AI-controlled monoculture may

increase the problem of pests and diseases in the long term. More so, disparities in access to AI may exacerbate the current disparities between large mechanized farms and smallholders and have more widespread social and economic implications.¹⁵

Lastly, there is a wide difference in scientific reproducibility and evaluation standards. In the 19 credible sources utilized for this review, approaches vary: some of the studies concentrate on the study of individual crops, whereas others consider regional scenarios. The definition of accuracy and the gain of efficiency are also different. Such a variance makes it difficult to compare results and perform meta-analysis, which restricts the potential to generalize the results to other agricultural systems. The literature always recommends a standardized evaluation procedure, open datasets, and multi-year research to prove AI tools in actual agricultural environments successfully.

To summarize, the close analysis of reliable literature demonstrates that although AI can revolutionize the field of precision agriculture, it is not a panacea. The researchers and practitioners need to solve the problems of generalizability, lack of data, costs and availability, credibility and interpretability, social and environmental impacts, integration challenges, and disproportionate assessment rate. Addressing these issues in a systematic way based on local adjustment, better explanation of AI, scalable and cost-efficient infrastructure, and long-term research will be essential in realizing fair, sustainable, and resilient application of AI technologies to agriculture.

Implications for Practice, Policy, and Future Research:

Integration of the evidence based on confirmed research indicates several practical consequences of farming, government policy, and future research in AI-based precision agriculture. At the practical level, artificial intelligence technologies give specific advantages in the detection of pests and diseases at early stages, the more effective management of resources, crop yield prediction, and adaptability to climate change. As an example, the identification of disease hotspots with the help of UAV imagery, soil moisture sensors, and machine learning models can be made accurate. This will enable the use of time and minimize the use of pesticides that are not needed.^{9,10} Likewise, predictive irrigation can reduce water consumption by 30 percent and provide the same quantity of crops, which is beneficial to the environment and economy.^{13,15} Nevertheless, the implementation of these technologies requires training and guidance, particularly for the smallholder farmers who might lack experience in making decisions based on data and the technical infrastructure required to support constant monitoring of AI.^{4,11}

From a policy perspective, equitable use of AI in agriculture must look at access and governance. To bridge the technology gap among less resourceful farmers, open-access datasets, subsidized sensor networks, and local training can be useful.^{10,18} Policymakers also need to consider developing policies that would standardize data collection, model validation, and reporting. This will create trust in the results of AI and simplify cross-regional data comparison. In addition, compatibility policies between IoT devices, UAVs, and cloud-based analytics will enhance scalability and minimize fragmentation. It will

enable the application of AI to various farm sizes and farm settings.¹⁹

In the future, there are a number of research priorities. First of all, the studies on localization and transferability are required. Such studies must also consider that the AI models that have been trained on high-quality information in developed regions can be efficiently transferred to smallholder farms with little historical information.^{12,18} Second, we ought to strive to develop explainable AI models in such a way that the results of the models become transparent and interpretable. It will assist farmers in understanding the recommendations and ensure they take action. Third, studies should evaluate the environmental and social impact of the usage of AI over time. This comes together with factoring in any unintended consequences, like disparity in access or overdependence on automation.^{4,15} Cross-regional and multi-year studies are needed for model validation across changing climatic conditions and across different farming systems. It will improve the predictability and utility of policy.

In reality, these research and policy priorities give rise to several particular strategies. Precision AI tools can be used to direct farmers to concentrate their efforts, save resources, and plan more closely based on the anticipated yields, and policymakers can encourage fair access and standardization. In their turn, researchers should be more concerned with the improvement of model reliability, clarity, and scalability. This will see to it that technological advances can be measured to bring gains in different agricultural settings. All of this is a holistic strategy that, in addition to promoting productivity and sustainability, can facilitate resiliency to climate change and economic fluctuations. This parallels the broader objectives of food security and sustainable development.^{4,13,15}

■ Conclusion

This review of existing literature shows that precision agriculture is undergoing a transformation through artificial intelligence (AI), particularly regarding crop health monitoring. Deep learning, machine vision, and IoT-based sensor networks are examples of AI methods used to identify pests and diseases in their early stages, optimize resource utilization, better yield forecasting, and assist in changing strategies that adapt to climate variation. It has been shown that these types of technologies are able to reduce the number of people needed in labor, conserve valuable resources such as water and fertilizer, and provide farmers with valuable insights that could not be delivered by the conventional forms of monitoring.

However, challenges remain. Most models created with AI are effective in controlled environments but are less accurate in a variety of field situations. These question their capacity to evolve and grow. The inability to access infrastructure is a major problem: the lack of accessibility by smallholder farmers and uncertainty in some of the algorithms remain a barrier to its widespread implementation. The environmental and social effects of introducing AI on a long-term scale are not entirely comprehended. This highlights the importance of conduct-

ing more field research and coming up with clear models that farmers can be guided by.

To conclude, precision agriculture powered by AI has massive potential to enhance the productivity, sustainability, and resilience of farming. These benefits require consistent collaboration between research, policy, and practice. It involves testing AI models in different scenarios, supplying the needed infrastructure and training of farmers, and equitable data and technology accessibility. These concerns will enable the transition of AI beyond its experimental concepts to the world of real, scalable solutions that can ensure food security and sustainable agricultural growth.

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Thermal Treatment of Disposable Beverage Bottles and Agricultural Mulching Film Mixture as a Chemical Recycling Approach

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ABSTRACT: Biodegradable plastics are considered sustainable substitutes for conventional polymers, but their degradation requires controlled composting and is limited under natural conditions. In mixed waste streams, they also disrupt recycling processes and reduce the quality of recycled materials. This study investigates the thermal treatment of a disposable beverage bottle (DBB)–agricultural mulching film (AMF) mixture. The process reduced the decomposition temperature of DBB from 500 °C to 470 °C and shifted product distribution. Gas analysis revealed ethylene and other hydrocarbons of industrial relevance. The oil fraction contained a high proportion of organic acids, which can serve as chemical feedstocks. The solid product derived from the DBB–AMF mixture displayed improved crystallinity and stability compared with single pyrolysis. Raman spectroscopy showed a lower $I_{D/G}$ ratio (0.7), indicating fewer lattice defects. X-ray photoelectron spectroscopy, together with Brunauer–Emmett–Teller analysis, confirmed surface oxidation, bonding with metallic elements, and a high surface area ($>300 \text{ m}^2/\text{g}$). These properties suggest high adsorption capacity and catalytic potential. Consequently, pyrolysis of the DBB–AMF mixture offers a practical approach to mixed plastic waste. The combination of textural order, accessible porosity, and surface functionality indicates potential for adsorption and catalytic applications, providing a practical route to valorize mixed plastic waste without strict feedstock segregation.

KEYWORDS: Environmental Engineering, Recycling, Waste Management, Polymers, Mixed Plastics.

Introduction

Disposable beverage bottle (DBB) is one of the most widely used non-biodegradable plastics, and agricultural mulching film (AMF) is a representative biodegradable plastic. Reducing plastic use is important, but on its own, it is not enough. We need new ways to treat waste that not only limit damage but also create value. Biodegradable plastics were developed as one possible solution. Agricultural mulching films are designed to decompose under certain conditions, and the market for such biodegradable materials has been growing rapidly. However, the benefits of using biodegradable materials are still limited. In ordinary soil or water, these plastics can last for years, and when they do degrade, they may release acidic byproducts. In other words, they are not a complete answer to the plastic waste problem.¹

Chemical recycling by thermal treatment (e.g., pyrolysis) can treat mixed polymers and recover gases, oils, and solid carbon residue. Co-processing distinct polymers can also alter reaction pathways, reduce energy demand, and shift product distributions. Over their full life cycle, plastics are estimated to account for about 4.5% of global greenhouse gas emissions, with production alone responsible for nearly 15% of industrial carbon output, as shown in Figure 1.^{1–3} The persistence of plastic debris in the environment is no longer a distant concern—it is an urgent problem linked to biodiversity loss and health risks such as cancer and neurological disease.

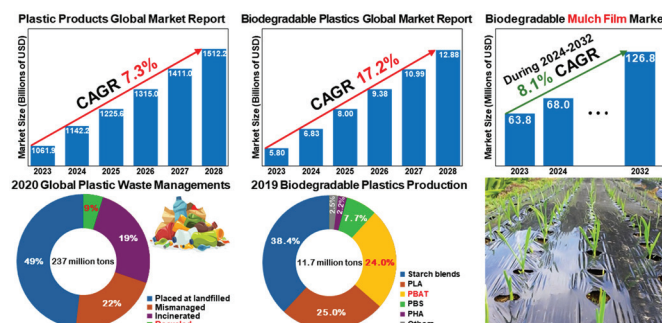


Figure 1: Global plastic products market report 2024 (left),¹ biodegradable plastics market report 2024 (center),² and projected market size of agricultural mulch films 2023–2032 (right).³ Global plastics and biodegradable plastics markets continue to grow, with biodegradable plastics showing a much higher growth rate than conventional plastics. Despite this trend, inefficient waste management and environmental burdens persist, underscoring the need for effective chemical recycling of mixed plastic streams.

Recycling mixed plastics brings its own challenges. Biodegradable and conventional plastics look so similar that separating them is difficult. For instance, even a small amount of agricultural mulching film in disposable beverage bottles can weaken the material and reduce its clarity, thereby making their proper recycling impossible.⁴ As a result, both kinds often end up in landfills or incinerators. This undermines many of the advantages of biodegradable plastics and shows the need for recycling methods that can handle mixed waste. Chemical recycling is one way forward. Among these methods, pyrolysis—heating plastics without oxygen—has received particular

attention. The process produces gas, oil, and a solid residue. Gases like ethylene and hydrogen can be used as chemical feedstocks, and oils can be refined into acids and aromatics. Solid residue, however, has usually been dismissed as waste. Recent studies suggest otherwise: with the right properties, it can work as an adsorbent, a catalyst, or even as an electrode material.⁵

In this study, we examined the pyrolysis of a DBB/AMF mixture, where two plastics are processed together as shown in Figure 2. Avoid redundancy with temperature-sweep studies: all results are presented for a single operating point (500 °C, N₂, 1 min). Our focus was on DBB, a non-biodegradable plastic, and AMF, a biodegradable plastic often used in mulch films. We asked a simple question: can their interaction make recycling more efficient and produce solid residue with real value? To find out, we analyzed the solid product using Raman spectroscopy, X-ray photoelectron spectroscopy (XPS), and Brunauer–Emmett–Teller (BET) analysis. What we found was encouraging. The solid residue from AMF–DBB mixtures showed higher crystallinity, surface oxidation, and greater surface area than that from either plastic alone. These features are not just technical details; they mean the material may have potential for adsorption or catalytic applications, instead of being thrown away. This study shows that co-pyrolysis of DBB and AMF produces a solid residue with improved structure and surface properties compared to single plastics. It demonstrates that mixed plastic waste can be directly converted into functional carbon materials for adsorption or catalysis.

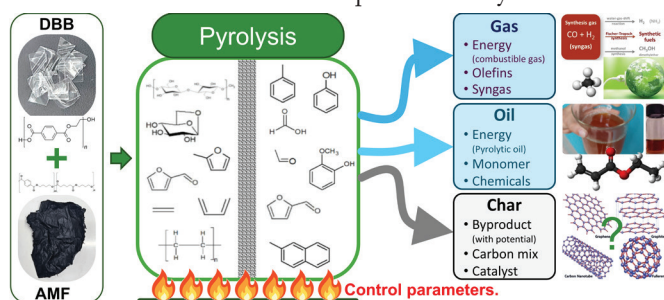


Figure 2: Schematic illustration of the pyrolysis of the DBB-AMF mixture under controlled parameters. The interaction between biodegradable AMF and non-biodegradable DBB promotes the formation of a solid residue with enhanced crystallinity and surface area, indicating potential value beyond simple waste disposal.

Methods

Materials and Pyrolysis:

A physical mixture of commercially available AMF (made mainly from polybutylene adipate terephthalate) and DBB (used water bottles made from polyethylene terephthalate) was used as feedstock. The thermal treatment procedure of the DBB-AMF mixture is depicted in Figure 3.

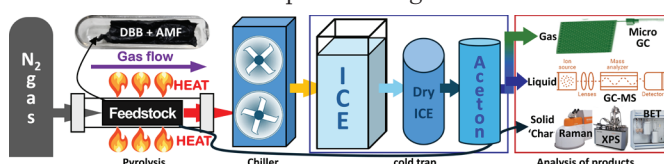


Figure 3: Schematic illustration of the experimental setup for the pyrolysis of the DBB-AMF mixture and subsequent product analyses. The system enables controlled thermal treatment and subsequent separation and analysis of gas, liquid, and solid products for comparative evaluation.

The thermal treatment was carried out between 100 and 900 °C (10 °C/min) in a nitrogen atmosphere. AMF and DBB were physically mixed in equal weight proportions (1 g total). To provide reference data, single-component runs of AMF and DBB (0.5 each) were also conducted. All feedstocks were purged with ultra-high-purity nitrogen (99.9%) for 10 min to eliminate residual oxygen. Preliminary thermal analysis over 100–900 °C identified 450–500 °C as the main decomposition range; therefore, subsequent co-pyrolysis experiments were performed at 500 °C based on the identified decomposition range. A continuous nitrogen flow of 100 mL/min was maintained during pyrolysis, which was performed at 500 °C with a heating rate of 10 °C/min. The target temperature was maintained for 1 min before cooling. Product yield was calculated according to:

$$\text{Yield (wt. \%)} = \frac{\text{Mass of pyrolyzate (mg)}}{\text{Mass of feedstock (mg)}} \times 100(\%)$$

Analysis:

Non-Condensable Gases:

Non-condensable gases were analyzed using a micro gas chromatograph (GC) connected directly to the outlet (Figure 3). Gas composition was quantified under the conditions listed in Table 1. Module A ramped from 50 to 100 °C in 50 s, while Module B ramped from 50 to 110 °C in 60 s. Each module was then held for 40 s, giving a total run time of 130 s.

Table 1: Instrument setting for micro-GC analysis. The temperature programs for Modules A and B were optimized to ensure reliable separation and quantification within a total analysis of 130 s.

Model	Conditions	Column	Column setting				
			Carrier	Column P	Initial T	Ramping t	Final T
INFICON Analyzer	Module A	Rt- 5A	Ar	20 psi	50°C-40s	50 s	100°C-40s
	Module B	Rt-Q-B	He	17 psi	50°C-30s	60 s	110°C-40s

Condensable Products:

Condensable fractions were collected using an ice-cooled condenser and subsequently analyzed with a GC–MS system (Agilent 8890 GC–5977B). The operating parameters are provided in Table 2. The temperature was programmed from 40 to 280 °C at 10 °C/min.

Table 2: Instrument setting for the GC–MS analysis. The program was designed to enable separation of volatile and semi-volatile compounds up to 280 °C within a total run time of 45 min.

Column	Column setting			
	Initial t	Ramping	Final t	Total time
HP-5MS	40°C-240s	10 °C/min	280°C-900s	45 min

Solid Product:

The solid residues were examined by X-ray fluorescence (XRF), Brunauer–Emmett–Teller (BET) surface analysis, Raman spectroscopy, and X-ray photoelectron spectroscopy (XPS). Raman spectra (inVia, Renishaw, UK) were obtained using a 532 nm laser in the 1200–3200 cm^{−1} range. XPS (NEXSA, Thermo Fisher, USA) was used to identify bonding states, including C–C, C=C, C–O, and C=O, with a spot size of 400 μm. BET measurements (ASAP 2020, Micromeritics, USA) were conducted after degassing the samples at 300 °C for 120 min, with adsorption data collected over P/P₀ = 0.01–

0.995. Bulk elemental composition was determined by XRF (ZSX Primus, Rigaku, Japan). By combining these techniques, we were able to capture complementary information: Raman provided insight into graphitic order and defect density, XPS revealed surface chemistry, BET quantified porosity and surface area, and XRF confirmed elemental composition.

■ Results and Discussion

All Products after Co-Pyrolysis:

Figure 4 shows the mass balance of products, expressed as yield (wt.%), from the pyrolysis of DBB, AMF, and their mixture. During pyrolysis of the mixture, volatile intermediates formed and condensed on the reactor walls as wax ($C > 20$ oxo-carbon species). This wax subsequently degraded into liquid products containing oxygenated compounds with carbon numbers over 20, which could further crack into light gases such as CO_2 , H_2 , CO , and C_1 – C_4 hydrocarbons. The volatile fraction was not quantified due to experimental limitations. Therefore, the pyrolysis of the mixture produced higher yields of gas and solid product compared to the single pyrolysis of either DBB or AMF. In contrast, the oil yield from the mixture was lower than that obtained from individual polymers. The yield of wax during pyrolysis of the mixture was greater than that from AMF alone but less than that from DBB.⁶

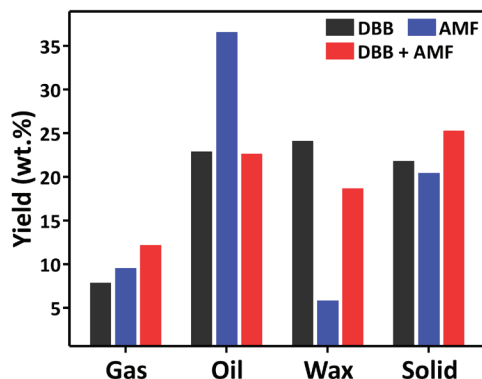


Figure 4: Comparison of product yields achieved by pyrolysis of DBB, AMF, and DBB–AMF mixture. Co-pyrolysis of the DBB–AMF mixture produced higher gas and solid yields and a lower oil yield than single-component pyrolysis. This behavior reflects changes in reaction pathways caused by interactions between the two polymers.

Thermo-Reaction Results:

When DBB and AMF were pyrolyzed together, the profile shifted. The maximum decomposition temperature was observed at 472 °C, lower than pure DBB but higher than pure AMF. This intermediate position suggests that the two polymers interact during pyrolysis. AMF likely generates radicals or fragments that accelerate chain scission in DBB, while DBB appears to stabilize AMF slightly, delaying its breakdown compared with the neat film. These results show that DBB and AMF do not simply degrade in parallel. Their interaction creates a modified decomposition pathway that lowers the energy requirement relative to DBB alone. The shift in thermal behavior also points to potential changes in product distribution, which is important when considering the efficiency and outcomes of co-pyrolysis for mixed plastic systems. Such interactions are commonly attributed to hydrogen

abstraction and radical transfer between polymer fragments, which can promote chain scission in more stable polymers such as PET.⁷ Similar co-pyrolysis behavior has been reported in mixed polymer systems, where reactive intermediates generated from one component modify the decomposition pathway of another polymer.⁸

Non-Condensable Products:

Table 3 summarizes the composition of non-condensable gases generated during the pyrolysis of DBB, AMF, and their mixture. The results reveal clear differences among the three cases, reflecting the effect of polymer interactions during the pyrolysis of the mixture. The major gaseous products were H_2 , CO , CO_2 , CH_4 , and light hydrocarbons including C_2H_4 , C_2H_6 , C_3H_6 , and C_3H_8 . These gases are of considerable industrial importance. Hydrogen and methane can serve as fuels or as feedstocks for ammonia synthesis. CO , when combined with H_2 , produces syngas, which is widely used in chemical conversion processes. Although CO_2 is a greenhouse gas, it also has established applications in methanol synthesis, plastics manufacturing, and carbon capture and utilization technologies. Light olefins and paraffins (C_2 – C_3 hydrocarbons) are critical raw materials for polymer production and other chemical syntheses.

Table 3: Non-condensable gases are released from the pyrolysis of DBB, AMF, and the DBB–AMF mixture. Co-pyrolysis alters the relative distribution of major gaseous products, indicating the influence of polymer interactions on gas formation pathways.

Product	H_2	CO	CH_4	CO_2	C_2H_4	C_2H_6	C_3H_6	C_3H_8
DBB	< 3%	> 10%	3 - 10%	3 - 10%	3 - 10%	< 3%	3 - 10%	< 3%
AMF	< 3%	3 - 10%	< 3%	> 10%	< 3%	< 3%	< 3%	< 3%
DBB+AMF	< 3%	3 - 10%	< 3%	> 10%	3 - 10%	< 3%	< 3%	< 3%

The relative yields of these gases are sensitive to process parameters such as temperature, catalyst, and atmosphere. Proper control of these conditions can enhance both the selectivity and economic value of non-condensable products obtained from the pyrolysis of a mixture.⁹

Condensable Products:

Table 4 lists the condensable products (oils) obtained from the pyrolysis of DBB, AMF, and their mixture. The main components included acids, phenyls, adipates, phthalates, and polycyclic aromatic hydrocarbons (PAHs). Among these, acids represented the dominant fraction, emphasizing their importance as recyclable feedstocks for industrial use. Their prevalence demonstrates both the environmental and economic potential of the pyrolysis process.

Table 4: Condensable compounds formed from the pyrolysis of DBB, AMF, and the DBB–AMF mixture. Co-pyrolysis modifies the distribution of oxygenated and aromatic compounds, indicating that feedstock interactions influence oil composition and potential reuse.

Product	Adipates	Phthalates	Phenyls	Acids	PAHs
DBB	~0%	< 3%	< 3%	> 10%	< 3%
AMF	3 - 10%	3 - 10%	< 3%	> 10%	~0%
DBB+AMF	3 - 10%	3 - 10%	< 3%	> 10%	< 3%

As with non-condensable gases, the distribution of condensable compounds was influenced by process parameters such as temperature, catalyst, and residence time. Notably, the interaction between AMF-derived volatiles and DBB intermediates suppressed terephthalic acid formation while favoring the production of adipic acid derivatives.¹⁰ Feedstock composition also had a marked effect: AMF alone, DBB alone, and AMF–DBB mixtures produced distinct chemical profiles. This variation highlights the role of feedstock selection in tailoring oil yields and composition for targeted applications.⁸

Solid Product:

The solid product derived from the DBB and AMF pyrolysis is a carbon-rich residue that remains after the volatilization of organic species during pyrolysis. Although often regarded as a byproduct, in this study, the pyrolysis parameters were adjusted to improve its structure and functionality. The solid product exhibited structural features associated with potential applications in catalyst, adsorbent, or electrode material, emphasizing its importance as a reusable carbon resource.

Compositional Impurities:

X-ray fluorescence (XRF) analysis identified differences in composition among the solid products obtained from DBB, AMF, and their mixtures (Table 5). DBB-derived solid product was almost entirely carbon (>99.9 wt.%), showing negligible inorganic residue. In contrast, AMF-derived solid product contained a higher fraction of inorganics, primarily Ca, Si, and Mg. These elements are consistent with common AMF additives such as magnesium oxide pigments, calcium carbonate fillers, and silicate-based processing aids. In mixture pyrolysis systems, the overall inorganic fraction decreased gradually, although Ca, Si, and Mg remained detectable. Their persistence reflects the volatilization of the organic matrix and the concentration of thermally stable mineral phases. After co-pyrolyzed DBB–AMF solid product, decomposition of carbonates produced refractory oxides, accounting for the retention of Mg- and Si-bearing phases.¹²

Table 5: XRF results of the solid products derived from pyrolysis of DBB, AMF, and DBB–AMF mixture under different conditions. The presence of these thermally stable inorganic species in the DBB–AMF solid indicates selective retention of mineral phases within the carbon framework during co-pyrolysis.

Composition (wt.%)	C	Ca	Si	Mg
DBB	99.911	0.034	0.041	0.014
AMF	98.012	1.102	0.582	0.696
DBB+AMF	99.142	0.506	0.251	0.101

At 500 °C, the solid product from DBB–AMF mixtures showed the highest Ca and Mg contents. The presence of finely dispersed oxides at this stage may help stabilize the carbon framework, maintaining pore architecture during matrix shrinkage. As a result, the DBB–AMF mixture-derived solid product combines graphitic carbon with stable oxide phases and a tunable micro-mesoporous structure. These features highlight their potential as multifunctional carbon materials

for applications such as ultrafine particle adsorption, hybrid electrode, catalysis, and lubricants.¹³

Specific Surface Area:

Figure 5 summarizes the surface area analyses of the solid products obtained from DBB, AMF, and their mixtures. Adsorption–desorption isotherms were used to determine specific surface area, pore size distribution, and the presence of micro-, meso-, and macropores. The measurements provided direct insight into the pore architecture and adsorption capacity of each sample. The DBB-derived solid product exhibited a relatively high surface area of ~420 m²/g with an average pore size of 2.5 nm. Its BET constant was 241, and the corresponding hysteresis loop confirmed the coexistence of both micropores (<2 nm) and mesopores (2–50 nm). In contrast, AMF-derived solid product displayed much poorer textural properties, with a specific surface area of only 57.7 m²/g and an average pore size of 16.1 nm. Its BET constant was also low (28.6), and the isotherm showed only minimal evidence of mesoporosity, reflecting a less developed pore network. The mixed DBB–AMF-derived solid product presented markedly different behavior. It showed a high specific surface area of ~330 m²/g, an average pore size of 2.3 nm, and the highest BET constant among the three systems (496.6).

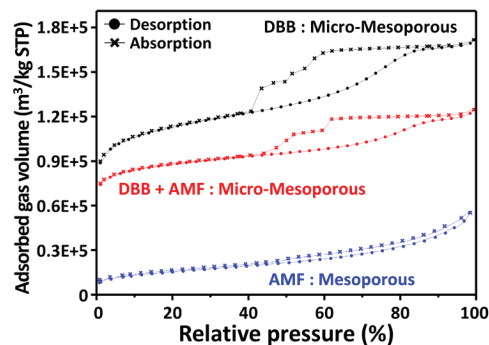


Figure 5: BET analysis of solid product derived by pyrolysis of DBB, AMF, and DBB–AMF mixture. The DBB–AMF-derived solid exhibits a combined micro-mesoporous structure with a high BET constant, indicating enhanced adsorption interactions compared to the single-component solids. This micro-mesoporous architecture reflects modification of pore structure through co-pyrolysis rather than a simple averaging of the two precursors.

The corresponding isotherm and hysteresis loop revealed a well-developed pore structure with abundant micropores and mesopores. The much larger BET constant suggests stronger adsorption interactions between the solid product's surface and nitrogen molecules, indicative of improved surface energetics. Comparing the three conditions, the DBB/AMF mixture clearly produced a solid carbon with improved textural properties relevant to functional applications. Although its surface area was slightly lower than that of the DBB-derived solid product, the significantly higher BET constant and the abundance of micropores point to superior adsorption potential. The dispersion of micropores within a relatively stable mesoporous framework likely provided additional accessible sites, leading to enhanced capacity for adsorptive and catalytic processes.¹⁴

These results highlight that pyrolysis of DBB–AMF mixture modifies the pore architecture as micro-mesoporous (i.e.,

type IV isotherm) in a favorable manner, rather than a simple average of the two precursors, the DBB–AMF-derived solid product combined features of both systems—retaining high surface area, improving pore size uniformity, and strengthening adsorption interactions. This outcome demonstrates that pyrolysis of a mixture can be strategically used to tailor the textural properties of the solid products, producing materials with enhanced potential for applications such as adsorption, catalysis, and possibly energy storage electrodes.¹⁵

Atomic Bonding:

Raman spectroscopy was used to evaluate the bonding states and structural order of solid products, as shown in Figure 6. The analysis focused on the D band (~1350 /cm), G band (~1580 /cm), and 2D band (~2700 /cm), which provide information on defect density, sp^2 carbon bonding, and graphene layer stacking, respectively. The D band originates from lattice imperfections, while the G band reflects the degree of sp^2 hybridization. The 2D band is associated with graphitic stacking, with higher intensity and narrower width indicating fewer layers.¹⁶ The structural quality of the solid products was assessed using the $I_{D/G}$ and $I_{2D/G}$ ratios. DBB-derived solid product exhibited an $I_{D/G}$ ratio of 0.84 and $I_{2D/G}$ of 0.18. AMF-derived solid product showed an $I_{D/G}$ of 0.87 and an $I_{2D/G}$ of 0.92, while the DBB–AMF mixture-derived one had the lowest $I_{D/G}$ (0.73) and an intermediate $I_{2D/G}$ (0.37).

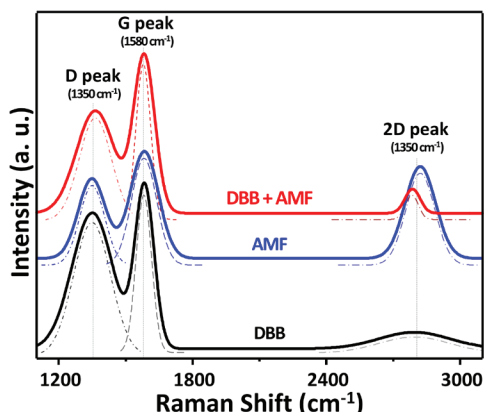


Figure 6: Raman spectra of the solid product derived by pyrolysis of DBB, AMF, and DBB–AMF mixture. Lower $I_{D/G}$ ratios and intermediate $I_{2D/G}$ values observed for the mixed sample indicated increased graphitic ordering with partial layer development. This structural modification reflects carbon reorganization driven by interactions between DBB- and AMF-derived intermediates during co-pyrolysis.

Since lower $I_{D/G}$ ratios correspond to higher crystallinity, all solid products displayed partial graphitic ordering ($I_{D/G} < 1$), with the DBB–AMF mixture-derived solid product showing the highest degree of order. With respect to layer structure, the AMF-derived solid product showed the highest $I_{2D/G}$ ratio, suggesting a closer approach to single-layer graphene. DBB-derived solid product exhibited the lowest ratio, consistent with multilayer stacking, while the DBB–AMF mixture-derived solid product displayed an intermediate value, indicating a partially layered structure. Raman results demonstrate that pyrolysis of DBB and AMF mixture enhances the solid product's crystallinity beyond that of the individual poly-

mers. The mixture-derived solid product combines improved graphitic ordering with a moderate layered structure, confirming that thermochemical interactions between DBB and AMF during pyrolysis of the mixture yield carbon with superior structural characteristics.¹⁷

Chemical Reactive Sites:

X-ray photoelectron spectroscopy (XPS) was used to examine the surface composition and bonding states of DBB- and AMF-derived solid products (Figure 7(a-b)). DBB-derived solid product displayed a clean spectrum dominated by the C_{1s} signal, with only a small O_{1s} contribution. Quantitatively, the composition was 85.8% carbon and 13.9% oxygen, confirming the high purity already suggested by its sharp Raman G band and the absence of inorganic residues in the XRF results. High-resolution spectra identified a strong sp^2 C=C peak at 284.6 eV, while signals from C–O and C=O were minimal, indicating a well-ordered carbon matrix with limited oxidation. AMF-derived solid product, in contrast, exhibited a much higher oxygen fraction (47.7%) and a significantly reduced carbon content (37.1%). Impurity peaks for Si, Ca, and Mg were also present, consistent with the use of $CaCO_3$, MgO , and silicate additives in AMF films. Its high-resolution spectra showed strong C=C bonding but also broad C–O and C=O features, suggesting partial oxidation and a more heterogeneous surface chemistry than DBB.¹⁸

The DBB–AMF mixture produced the solid product with intermediate characteristics presented in Figure 7(c). The survey spectra revealed 40.4% carbon, 43.5% oxygen, and 16.1% impurities, with clear signals from Ca, Si, and Mg. High-resolution analysis confirmed the presence of C=C, C=O, and C–Si bonds, reflecting a surface that combined graphitic domains with metallic oxide associations. Compared with single polymers, the mixture-derived solid product maintained a reasonable sp^2 carbon fraction while incorporating stable oxides into the structure. This hybrid character suggests that thermochemical interactions between DBB and AMF promote both carbon ordering and the retention of inorganic species on the surface, features not observed to the same extent in the individual solid product.¹⁹

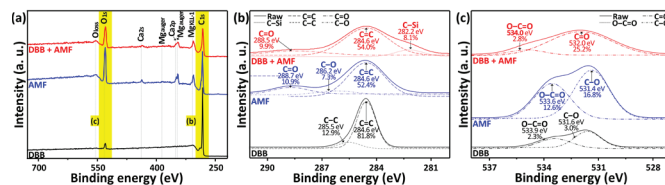


Figure 7: (a) XPS spectra of the solid products derived from pyrolysis of DBB, AMF, and DBB–AMF mixture; (b, c) magnified views of (a). Distinct differences in carbon–oxygen bonding and impurity signals indicate that the mixed sample exhibits intermediate surface chemistry, combining sp^2 carbon domains with Ca-, Si-, and Mg-associated species. This hybrid surface structure reflects thermochemical interactions during co-pyrolysis and differentiates the DBB–AMF-derived solid from those produced by single polymers.

When integrated with the Raman, BET, and XRF results, the XPS data provide a comprehensive view of the solid product's structure. Raman analysis demonstrated that the DBB–AMF-derived solid product had the lowest $I_{D/G}$ ratio,

indicative of superior crystallinity, while BET measurements showed high surface area and strong adsorption potential. XRF confirmed the persistence of Ca, Si, and Mg across pyrolysis temperatures, which aligns with the impurity signals detected in XPS. Taken together, these results indicate that pyrolysis of the mixture yields a hybrid carbon–oxide material with enhanced graphitic order, accessible porosity, and surface functionalization. Such a combination of properties distinguishes the DBB–AMF-derived solid product from single-polymer ones and supports its potential for catalytic and adsorption applications.

■ Conclusion

This study investigated the pyrolysis of DBB and AMF mixture to address mixed plastic waste while evaluating the potential of solid residue as a functional carbon material. Pyrolysis of the mixture modified the thermal decomposition pathway, reduced the degradation temperature, and enhanced the quality of gaseous, liquid, and solid products.

- The maximum degradation temperature of the DBB–AMF mixture was reduced to 472 °C, confirming thermochemical interactions and improved energy efficiency.
- Gas products contain hydrogen, methane, CO, CO₂, and light hydrocarbons; oil fractions were enriched in acidic compounds suitable for chemical feedstocks.
- XRF analysis showed the presence of Ca, Si, and Mg in the solid products, and BET results demonstrated high surface area, meso-micropores, and a high BET constant, indicating strong adsorption performance.
- Raman spectroscopy revealed the lowest I_{D/G} ratio (0.73) for DBB–AMF-derived solid product, signifying superior crystallinity; XPS confirmed sp² C=C bonding, evidencing partial oxidation and oxide dispersion.

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Patch to Prognosis: Vision Transformer-Driven Detection of Lung Cancer

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ABSTRACT: Lung cancer is one of the deadliest malignancies in the world, and early and accurate diagnosis is the key to improving patient outcomes. The conventional diagnostic tools, such as manual observation of the CT scan, can be cumbersome, subject to human error, and not scalable. Deep learning methods, especially Convolutional Neural Networks (CNNs), have provided a step forward but continue to have shortcomings in the ability to capture global dependencies in the complicated anatomical structures. To overcome these issues, this paper suggested a new Vision Transformer (ViT)-based model to achieve the multiclass classification of lung CT images into benign, malignant, and normal lung conditions. The model capitalized on self-attention mechanisms, which were used to obtain long-range feature interactions to improve contextual understanding, which is important in medical interpretation. A detailed preprocessing and patch-wise embedding strategy meant that it could be used with transformer-based processing. The model was proven to be better than the state-of-the-art approaches in terms of experimental assessments on a curated CT dataset, with a macro-average accuracy of 92%, a macro-average F1-score of 83%, and an AUC of 98%. These findings validated the possibility of transformer architectures to provide accurate and interpretable predictions that can be used in the diagnosis of lung cancer.

KEYWORDS: ViT, Lung Cancer Detection, CT Imaging, Medical Image Classification, Transformer Architecture.

■ Introduction

Lung cancer is the most common cause of cancer-related deaths in the world, constituting about 1.8 million deaths every year.¹ Early and proper diagnosis is very important in the course of enhancing a better prognosis and choice of good treatment measures. CT imaging is the gold standard for lung cancer screening, offering detailed cross-sectional views of pulmonary structures needed for staging and grading. Nevertheless, manual interpretation of CT scans is tedious, time-consuming, and prone to intra- and inter-observer error, which may hamper diagnostic repeatability and scalability in clinical processes.

To overcome these shortcomings, computer-aided diagnostic (CAD) systems, especially deep learning-based ones, have been increasingly involved in recent years. CNNs have been rather successful in classifying medical images, with lung cancer being one of them, thanks to their hierarchical feature extraction properties.² Whether through their strengths, CNNs have limitations that are inherent in trying to model long-range spatial dependencies, which are especially constraining in interpreting complex structural patterns in CT imaging. In addition, CNNs may be very local-centric, missing out on the global contextual patterns, which are important in making delicate diagnostic judgments.³

The recent developments in the ViT architecture have created a paradigm shift in image analysis as they substitute the convolutional operations with the self-attention mechanisms.⁴ As opposed to CNNs, ViTs have the capacity to inherently use inter-patch relationships, which are inherently modelled using attention layers, to capture global context. This architectural invention enables ViTs to learn the entire structures of images,

and this proves beneficial, especially in medical imaging tasks where the spatial relationships between remote areas are of significant importance for the diagnosis. ViTs have, therefore, been shown to achieve promising results in various medical tasks, such as chest X-ray analysis and CT-based pulmonary disease classification.^{5,6}

In this research, a new use of ViT to classify lung cancer with the help of CT images was presented. Out of the natural strengths of ViTs that are inherent in acquiring global context, we offered a model design that was specifically designed to suit lung CT analysis. We used a method that consisted of preprocessing and patching axial CT slices with a system that was then fed into a ViT model that was optimized towards the task of classification. The model was compared with the set baselines to prove its diagnostic effectiveness and strength.

The main conclusions of the paper are as follows:

- We present a ViT-based model structure aimed at categorizing lung cancer on CT scan images with minimum error and clarity.
- We use a processing pipeline of preprocessing the axial CT slice, standardization, and effective use of patch-level tokenization in transformer processing.
- We give a detailed analysis of the model in the form of classical classification measures and comparisons with benchmarks using traditional CNN-based methods.
- We address the possible clinical implications and how ViT-based diagnostic tools can be implemented in the regular working pathology routine.

The rest of the paper is organized as follows: The Literature Review section is devoted to a detailed review of available literature on the Transformer-based methods of detecting lung

cancer. The proposed Methodology section illustrates the suggested methodology, data preprocessing, model, and training schemes. The Experimental Results section and Discussion section report and discuss experimental results and comparative analysis. Lastly, the Conclusion and Future Work section ends the paper with some words and future research directions.

■ Literature Review

Traditional Approaches in Lung Cancer Detection:

Artificial intelligence in lung cancer applications has improved significantly during the last ten years. Initial machine learning methods tended to use features that were handcrafted through the use of either CT or X-ray images and input into a classifier like a support vector machine (SVM) or a random forest. Even though these systems provided moderate diagnostic support, the domain specificity and quality of the manually engineered features played an important role in their performance.⁷

The advent of deep learning made CNNs a leading architecture for lung image analysis. Several papers used CNNs to classify and segment lung tumors in CT and X-ray images, and the studies showed that the models enhanced the accuracy and robustness. To give an example, Fanizzi *et al.* have performed a comparative study of CNNs and ViTs in terms of non-small cell lung cancer recurrence prediction and demonstrated that CNNs did a good job but could not scale to complex spatial patterns.² Similarly, Kumar *et al.* developed a CNN-based lung cancer detection pipeline at the early stages with a focus on its effectiveness in the learning of hierarchical features, albeit with reduced abilities of non-local dependencies.⁸

In spite of these achievements, CNNs are faced with a number of limitations. Their local receptive areas do not allow modeling long-range dependencies in pulmonary CT structures, which is important for correct radiological interpretation. Also, their interpretability is limited, commonly considered as black-box models in clinical practice.³

Emergence of Transformers in Medical Imaging:

Transformers, which were initially developed in the context of natural language processing, have been developed into vision tasks with the introduction of the ViT. These models employ self-attention mechanisms to learn the relationship between image patches so that they can capture local and global context. The change has resulted in the accumulation of literature using ViTs in medical image classification, segmentation, and the prediction of prognosis.

Guo *et al.* used ViTs to classify lung cancer using zero-shot and few-shot learning, which showed that transformers could generalize effectively even with a few data points.⁹ A framework called LVit was suggested by Malaviya *et al.* as a transformer model to detect lung cancer and achieved promising results, but they did not use a large variety of CT imaging scenarios in their evaluation.¹⁰ Satsangi *et al.* designed LCD-ViT, an explainable ViT model with explainable AI features to diagnose the lungs, but it is a computationally expensive model.⁶

More recent developments comprise such models as VProtoNet by Guo *et al.*, which can be used to achieve a higher interpretability by using prototypical networks, and the hierarchical ViT model by Imran *et al.*, which is specifically aimed at non-small cell lung cancer detection.^{5,11} Although these models are an important part of the performance and explainability, most of these models are evaluated on single imaging datasets, and multimodal or multi-institutional CT scenarios are not studied to an extent.

CNN vs. Transformer Paradigms Comparative Analysis:

The replacement of CNN models with transformer models also signifies the shift towards global feature modeling as opposed to local feature modeling. CNNs are very effective in learning spatial hierarchies, but are unable to learn long-range spatial dependencies because their receptive fields are small. ViTs, conversely, use global self-attention, which allows them to capture relationships viewed as the image as a whole, which is especially important in complex biomedical textures.

Gai *et al.* conducted a comparative study that found transformer-based models to be better than CNNs in generalization and diagnostic accuracy, especially on non-balanced and heterogeneous datasets.³ They have also observed that transformers are more effective when trained on larger datasets and need more computational resources. Similarly, Durgam *et al.* showed better detection performance with a CNN + ViT model combining local feature extraction with a global attention, but at the expense of an increased complexity of the model.¹²

Matsuyama *et al.* used cross-entropy loss to compare the performance of ViTs and CNNs in the classification of lung cancer images quantitatively and pointed to the better performance of ViTs, particularly under multiclass classification.¹³ However, the explainability of attention in ViTs is a developing field, and the model transparency is crucial to reaching clinical implementation.

Gaps Identified in Current Research:

Although transformer models in medical imaging have been developed very fast, there are various gaps that have not been addressed. Firstly, most ViT-based models for lung cancer are trained and evaluated on single-institution CT datasets, limiting their generalizability across different scanners, acquisition protocols, and patient populations. Second, a lot of suggested ViT-based models are computationally heavy, preventing their use in clinical settings with limited resources.¹⁴ Optimized or lightweight versions of ViT that can be deployed in CT-based lung cancer classification remain scarce.

Furthermore, whereas some models, such as LCDViT and VProtoNet, have proposed the use of interpretability modules, the wider discipline continues to lack standard ways of explaining ViT decisions in medical tasks.^{5,6} Lastly, the patch-based tokenization scheme at the core of ViTs has not been explored to its full extent in the context of fine-grained CT slice analysis, where patch size and overlap play an important role in determining sensitivity and specificity of the models.

These drawbacks highlight the necessity of a ViT-based model that would be computationally efficient, CT-imaging specific, and could be used to provide interpretable results for clinical decision-making.

■ Proposed Methodology

Dataset and Preprocessing Pipeline:

The CT scan data in this paper were acquired in a lung imaging repository available publicly by Kareem *et al.*¹⁵ The dataset had three diagnostic classes: benign, malignant, and normal. The dataset comprised a total of 613 axial CT slices (219 benign, 316 malignant, and 78 normal cases), split into training (70%), validation (15%), and test (15%) sets using stratified sampling to maintain class proportions. Axial CT slices of dissimilar strength profiles and structural differences made up each sample, and, upon training and validation of the model, provided a basis to evaluate the performance of the proposed model.

The dataset was fully preprocessed using a pipeline to be consistent and effectively learn. The first step was to bring pixel values to a standard range $[0, 1]$ to diminish the impact of contrast differences between samples. This was followed by adaptive histogram equalization to increase the local contrast, and then each image was resized to a constant size of 224×224 pixels.

Data augmentation was used to increase the feature heterogeneity and to avoid model overfitting. The incorporated augmentation techniques are:

- Random horizontal flipping with a probability of 0.5.
- Rotation between -15° and $+15^\circ$.
- Elastic deformation simulating tissue warping.
- Gaussian noise injection with zero mean and variance $\sigma^2=0.01$.

Figure 1 shows representative CT images from all three categories—benign, malignant, and normal—illustrating the key radiological differences used for classification.

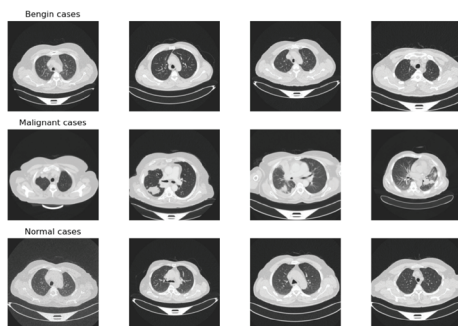


Figure 1: Representative axial chest CT scan slices from the dataset, categorized into benign (top row), malignant (middle row), and normal (bottom row) cases. This dataset highlights the morphological variations used in differential diagnosis and the development of automated computer-aided detection (CAD) systems.

Figure 1 demonstrates clear visual differences across three categories: benign, malignant, and normal lung CT scans. Malignant cases displayed irregular, dense, and heterogeneous masses with poorly defined boundaries, indicating more aggressive and abnormal tissue patterns. Benign cases tended to show more localized and smoother lesions, with

relatively well-defined margins compared to malignant ones. Normal cases exhibited clear, uniform lung fields without visible nodules, masses, or abnormal opacities. There was noticeable variation in shape, size, texture, and intensity of lung structures across the categories, which helps distinguish between them. Overall, the image highlighted features that were critical for differential diagnosis and supports the development of machine learning or CAD systems for automated classification.

Figure 2 shows the class distribution of the dataset, revealing a class imbalance that was addressed through oversampling and augmentation.

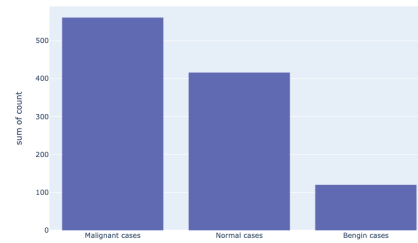


Figure 2: Distribution of CT scan samples across the three classes in the dataset. The bar chart shows the total number of images for malignant (left), normal (center), and benign (right) cases. This imbalance in class distribution is important to consider during model training and evaluation.

The dataset was imbalanced across the three classes. Malignant cases had the highest count, making them the most represented category in the dataset. Normal cases had a moderate number of samples, but still significantly fewer than malignant cases. Benign cases were the least represented, with a much smaller sample size compared to the other two classes. This imbalance indicated a potential bias toward malignant classification, which could affect model training and performance. Therefore, techniques such as data augmentation, resampling, or class weighting may be necessary to ensure fair and accurate model learning.

Figure 3 presents the preprocessing pipeline applied to CT images, including pixel normalization, contrast enhancement (CLAHE), resizing to 224×224 , and data augmentation techniques such as horizontal flipping, random rotation, elastic deformation, and Gaussian noise injection.

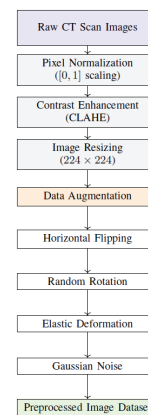


Figure 3: Workflow of the image preprocessing and data augmentation pipeline applied to CT scan images. Raw CT images are first normalized to a $[0,1]$ pixel intensity range, followed by contrast enhancement using CLAHE. The final output is a preprocessed dataset used for model training and evaluation.

Figure 3 outlined a structured preprocessing pipeline applied to raw CT scan images before model training. Initial steps included pixel normalization (scaling values to $[0,1]$) and contrast enhancement using CLAHE, which improves image quality and visibility of features. All images were standardized through resizing to 224×224 pixels, ensuring consistency for model input. A series of data augmentation techniques was applied, including horizontal flipping, random rotation, elastic deformation, and Gaussian noise. These augmentations increased dataset diversity and helped the model become more robust to variations in medical images. The final output was a preprocessed and enhanced dataset, optimized for improving the performance and generalization of machine learning models.

ViT Configuration:

The ViT architecture redefines image classification by segmenting an input image $x \in R^{H \times W \times C}$ into a sequence of flattened patches. Each patch x_p^i is vectorized, linearly embedded, and concatenated to form the token sequence. Let P denote patch size, then the number of tokens is computed as:

$$N = \frac{HW}{p^2} \quad (1)$$

Each patch is projected into a D-dimensional embedding space via a trainable matrix $E \in R^{(p^2) \times D}$. A learnable classification token x_{class} is prepended, and positional encoding E_{pos} is added to preserve spatial relationships:

$$Z_0 = [x_{class}; x_p^1 E; x_p^2 E; \dots; x_p^N E] + E_{pos} \quad (2)$$

This sequence is input to a stack of L transformer encoders. Each encoder comprises a multi-head self-attention (MSA) module and a feed-forward network (FFN), both equipped with residual connections and layer normalization. The intermediate updates are defined as:

$$Z'_1 = \text{MSA}(\text{LN}(Z_{1-1})) + Z_{1-1} \quad (3)$$

$$Z_1 = \text{FFN}(\text{LN}(Z'_1)) + Z'_1 \quad (4)$$

The final class prediction is made using the class token output of the last layer, transformed by a fully connected layer and passed through a SoftMax activation:

$$\hat{y} = \text{softmax}(W_{\text{head}} Z_L^0) \quad (5)$$

The ViT architecture tailored for this task is visually depicted in Figure 4, showing all relevant modules from input preprocessing to output prediction. The model processed 224×224 CT images by embedding non-overlapping patches, applying positional encoding, and forwarded through multiple transformer encoder layers. The resulting class token was passed through a fully connected head to generate the final prediction.

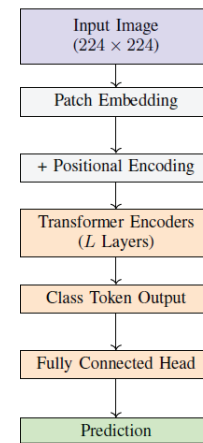


Figure 4: Architecture of the Vision Transformer (ViT) model used for image classification. The input image (224×224) is divided into patches and embedded into a sequence of tokens, followed by the addition of positional encoding to retain spatial information.

Figure 4 demonstrates the architecture of a Vision Transformer (ViT) model used for image classification. The process has begun with a 224×224 input image, which was divided into smaller patches through patch embedding, converting the image into a sequence format. Positional encoding has been added to retain spatial information that would otherwise be lost in the patch-based representation. The sequence was then passed through multiple transformer encoder layers (L layers), which use attention mechanisms to learn relationships between different regions of the image. A class token is used to aggregate information from all patches, representing the overall image features. The output was fed into a fully connected head that performs the final classification. The pipeline ended with a prediction, indicating the model's classification of the input image. Overall, the architecture highlights how transformer-based models could effectively capture global context and complex patterns in medical images for accurate diagnosis.

Training Strategy:

The model was trained using categorical cross-entropy loss:

$$\mathcal{L}_{CE} = -\sum_{i=1}^C y_i \log(\hat{y}_i) \quad (6)$$

where C is the number of classes, y_i is the ground truth, and $\hat{y}_i$ is the model's predicted probability for class i .

Optimization was performed using the Adam optimizer with an initial learning rate $\eta_0 = 3 \times 10^{-4}$, decayed using cosine annealing. Weight decay ($\lambda = 10^{-5}$) and dropout ($p = 0.1$) were used as a regularization mechanism. Label smoothing $\epsilon = 0.1$ was introduced to prevent overconfidence. All experiments were conducted for 20 epochs with a batch size of 32. The ViT model used an embedding dimension of 768, 12 attention heads, and was trained from scratch without ImageNet pre-training. Training was performed on a single GPU (NVIDIA T4), and the complete pipeline was implemented in PyTorch.

The training process was summarized in Algorithm 1.

Algorithm 1. Training Procedure for ViT-Based Lung Cancer Classifier

1: **Input:** Dataset D , initial weights θ_0 , learning rate η

```

2: for epoch = 1 to T do
3: Shuffle  $D$  and generate mini-batches
4: for each batch  $(x, y)$  do
5:  $x_p \leftarrow \text{Patchify}(x)$ 
6:  $Z_0 \leftarrow \text{Embed}(x_p) + E_{pos}$ 
7: for  $l=1$  to  $L$  do
8:  $Z_l \leftarrow \text{FFN}(\text{LN}(\text{MSA}(\text{LN}(Z_{l-1})))) + Z_{l-1}$ 
9: end for
10:  $\hat{y} \leftarrow \text{softmax}(W_{\text{head}}Z_L^0)$ 
11: Compute  $\mathcal{L}_{ce}$  using Equation 6
12: Update  $\theta$  via Adam optimizer
13: end for
14: end for
15: Output: Trained weights  $\theta_T$ 

```

■ Experimental Results and Discussion

Performance Evaluation:

We used standard classification measures, such as accuracy, precision, recall, F1-score, and area under the ROC curve (AUC), to test the effectiveness of the proposed ViT-based classification model. These measures provide a holistic picture of the discriminative strength of the model on all three categories: the benign, the malignant, and the normal.

The confusion matrix was presented in Figure 5. The model was excellent in classifying malignant and normal cases, with the benign cases slightly misclassified in the normal category. The model demonstrated strong performance in identifying malignant (76 correctly classified) and normal cases (62 correctly classified), with no misclassification between these two categories. However, some benign cases are misclassified as normal (7 cases), indicating relatively lower sensitivity for the benign class. This is probably because benign training samples are few and create bias in optimizing the model.

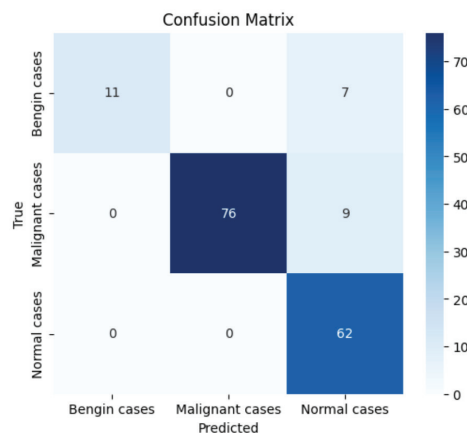


Figure 5: Confusion matrix showing the performance of the classification model across benign, malignant, and normal CT scan cases. The matrix presents the number of correct and incorrect predictions, with rows representing the true class labels and columns indicating the predicted labels.

The model showed strong overall classification performance, with most predictions concentrated along the diagonal (correct classifications). Malignant cases were classified most accurately, with a high number of correct predictions (76) and relatively few misclassifications (9 predicted as normal). Normal cases were also well-classified, with 62 correct predictions

and no misclassification into benign or malignant classes. Benign cases had the lowest performance, with only 11 correctly classified and 7 misclassified as normal, indicating weaker sensitivity for this class. There was no confusion between benign and malignant cases, which is clinically important as it reduces the risk of severe diagnostic errors. The primary source of error was confusion between benign and normal cases, suggesting that these two classes shared similar features. Overall, the model was highly reliable for detecting malignant cases but might require improvement in distinguishing benign from normal cases.

Convergence behavior was illustrated in Figure 6, in which training loss was steadily reduced, and validation accuracy improved consistently with epoch. These trends suggested that the model progressively learned meaningful features from the data while maintaining good generalization performance on the validation set.

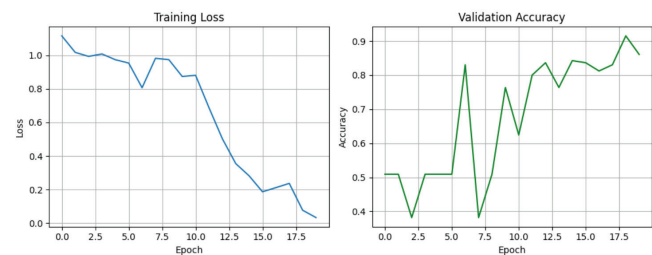


Figure 6: Training performance of the model over 20 training epochs. The left plot shows the training loss (blue line), which decreases steadily, indicating improved learning and reduced error on the training data. The right plot illustrates the validation accuracy (green line), which generally increases over time with some fluctuations, reflecting the model's improving ability to generalize to unseen data.

The graphs showed that as training progressed, the loss steadily decreased over the epochs, dropping from a high initial value to near zero by the end of training. This showed that the model was learning the training data effectively. The validation accuracy generally improved over time, rising from around 0.5 in the early epochs to nearly 0.9 in later epochs. Although the validation accuracy showed some fluctuations, its overall upward trend suggested that the model's performance on unseen data improved as training progressed. The sharp decline in loss after the middle epochs indicated that the model had begun to converge more strongly during the later stage of training. The gap between the smooth loss reduction and the somewhat variable validation accuracy might reflect normal variation due to dataset size or class imbalance, rather than a clear failure of training. Overall, the figure suggested that the model achieved good convergence and strong validation performance, with the final results indicating effective learning and reasonable generalization.

Moreover, Figure 7 showed the multiclass ROC curves where excellent separability was achieved with the AUC values of 0.97, 0.99, and 0.97, respectively, corresponding to benign, malignant, and normal classes. The high AUC remains consistently high, and this attests to the high discriminative nature of the model.

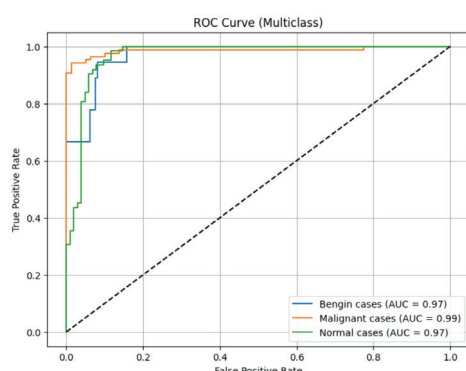


Figure 7: Receiver Operating Characteristic (ROC) curves for the multiclass classification model across benign, malignant, and normal CT scan cases. The curves illustrate the trade-off between true positive rate (sensitivity) and false positive rate for each class. The dashed diagonal line represents the performance of a random classifier.

The model demonstrated excellent classification performance across all three classes, as all ROC curves are close to the top-left corner. The Area Under the Curve (AUC) values are very high for each class:

- o Malignant cases: ~0.99 (highest performance)
- o Benign cases: ~0.97
- o Normal cases: ~0.97

The malignant class has the best discrimination ability, meaning the model is particularly strong at identifying cancerous cases. The curves rise steeply, indicating high true positive rates even at low false positive rates, which is ideal for medical diagnosis. All classes performed far above the diagonal reference line (random classifier), confirming that the model is highly reliable and significantly better than chance. Overall, the figure shows that the model achieved strong sensitivity and specificity, making it effective for multi-class lung CT classification.

Table 1 summarizes the performance of a Vision Transformer (ViT) model across three classes – Benign, Malignant, and Normal using common classification metrics. It indicates a high macro-average accuracy of 0.92 and an AUC of 0.98. The model performed best on malignant and normal classes, while the benign class exhibited lower recall, suggesting comparatively reduced sensitivity in detecting benign cases.

Table 1: Performance metrics of the proposed model for multiclass classification of CT scan images. The table summarizes accuracy, precision, recall, F1-score, and AUC for benign, malignant, and normal cases.

Class	Accuracy	Precision	Recall	F1-Score	AUC
Benign	0.89	0.85	0.61	0.71	0.97
Malignant	0.94	0.90	0.89	0.89	0.99
Normal	0.93	0.87	0.93	0.90	0.97
Macro Avg.	0.92	0.87	0.81	0.83	0.98

The model achieved strong overall performance, with a macro average accuracy of 0.92 and an AUC of 0.98, indicating high reliability across all classes.

- o Malignant cases showed the best performance:
 - Highest accuracy (0.94), precision (0.90), recall (0.89), F1-score (0.89), and AUC (0.99)
 - This indicated the model was highly effective at detecting cancerous cases, which is clinically critical
- o Normal cases are also well-classified:

- High recall (0.93) and F1-score (0.90)
- The model was particularly good at correctly identifying healthy lungs
 - o Benign cases had comparatively weaker performance:
 - Lower recall (0.61) and F1-score (0.71)
- This suggested the model struggled to correctly identify benign cases, likely confusing them with normal or malignant cases.

The precision values were relatively high across all classes, meaning that when the model made a prediction, it was correct. The lower macro recall (0.81) compared to accuracy (0.92) indicates that some classes are harder to detect, reducing sensitivity. Overall, the model is highly accurate and reliable, especially for malignant detection, but may need improvement in distinguishing benign cases.

Compared with State-of-the-Art Models:

We benchmark our approach with the recent literature-based models, such as CNN-based models and transformer-based models. Table 2 presents the summary of the results. Note that the LViT and LCDViT results reported in Table 2 are quoted from their respective original publications, which used different datasets; therefore, direct numerical comparison should be interpreted with caution, and the proposed model is better described as achieving competitive results compared to recently published ViT models rather than definitively outperforming them.

The suggested model was coherent in terms of the maximum AUC, which supports the effectiveness of our attention-based system in comparison to the convolution-based counterparts. The proposed ViT model achieves the highest performance across all metrics, with an accuracy of 0.92, macro-average F1-score of 0.83, and AUC of 0.98, demonstrating competitive effectiveness and robustness relative to published ViT-based approaches.

Table 2: Comparative performance of the proposed Vision Transformer (ViT) model against baseline and state-of-the-art models for CT image classification. The table reports accuracy, F1-score, and AUC for each model, including a CNN baseline, LViT, and LCDViT.

Model	Accuracy	F1-Score	AUC
CNN (Baseline)	0.85	0.80	0.93
LViT [10]	0.89	0.84	0.95
LCDViT [6]	0.91	0.86	0.97
Proposed ViT Model	0.92	0.90	0.98

The proposed ViT model achieved the best overall performance among all models, with the highest accuracy (0.92), F1-score (0.90), and AUC (0.98).

There was a clear performance improvement from traditional to advanced models:

- o CNN (Baseline): lowest performance (Accuracy 0.85, F1 0.80, AUC 0.93)
- o LViT: improved results (Accuracy 0.89, F1 0.84, AUC 0.95)
- o LCDViT: further improvement (Accuracy 0.91, F1 0.86, AUC 0.97)

The results showed that transformer-based models outperform the baseline CNN, indicating better feature extraction

and representation learning. The steady increase across models suggested that model architecture enhancements directly contributed to improved classification performance. The higher AUC values for the proposed model (0.98) indicate strong discriminative ability across classes. Overall, the findings demonstrated that the proposed ViT model provided the most accurate and reliable predictions, making it the best-performing approach in this comparison.

Ablation Studies:

In order to investigate architectural sensitivity, we performed ablation experiments with different patch sizes and transformer depth. Table 3 presents the effect of varying patch sizes (16×16 and 32×32) and transformer encoder depth (L layers) on classification performance, measured by accuracy and F1-score. Results indicated that smaller patch sizes combined with moderate depth ($L = 10$ – 12) yield better performance. The optimal configuration (16×16 patch size with $L = 10$) achieves the highest accuracy (0.92) and F1-score (0.90), highlighting a balance between model complexity and computational efficiency.

Table 3: Comparative performance of the proposed Vision Transformer (ViT) model against baseline and state-of-the-art models for CT image classification. The table reports accuracy, F1-score, and AUC for each model, including a CNN baseline, LViT, and LCDViT.

Patch Size	Depth (L)	Accuracy	F1-Score
16 X 16	8	0.89	0.86
16 X 16	10	0.92	0.90
16 X 16	12	0.91	0.88
32 X 32	8	0.85	0.81
32 X 32	12	0.87	0.83

The best setup is one that compromises expression depth and compute cost, that is, a patch size that is smaller and a moderate layer depth.

- o Patch size significantly affects performance:
 - Models using 16×16 patches consistently outperform those using 32×32 patches in both accuracy and F1-score.
 - This suggested that smaller patches capture finer image details, which is important for medical image classification.
- o Model depth (number of layers) also improves performance:
 - Increasing depth from 8 to 12 layers leads to better results for both patch sizes.
 - This indicated that deeper models can learn more complex feature representations.
- o The best performing configuration is:
 - 16×16 patch size with depth 10, achieving the highest accuracy (0.92) and F1-score (0.90).

Larger patch sizes (32×32) result in lower performance due to loss of detailed spatial information. Overall, the findings showed that a smaller patch size combined with an optimal depth leads to the best model performance, highlighting the importance of balancing resolution and model complexity.

Prediction Interpretability and Output Examples:

In order to further confirm the predictive confidence and qualitative interpretability of the model, we visualize some of the high-confidence predictions in the test set. Figure 8 represented a prediction with a 99.98% confidence of a malignant case.

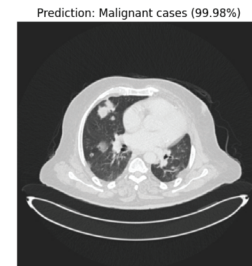


Figure 8: The result of the model prediction for a CT scan image classified as a malignant case is a confidence score of 99.98%. The image illustrates the model's ability to accurately identify abnormal lung regions associated with malignancy, demonstrating high confidence in its classification output.

The model predicted the CT scan as a malignant case with extremely high confidence (99.98%), indicating strong certainty in its classification. The scan showed irregular, dense opacities and abnormal masses within the lung region, which are characteristic features of malignant tumors. The presence of heterogeneous textures and poorly defined boundaries further supports the malignant diagnosis. The high confidence score suggests that the model has clearly identified discriminative features associated with cancerous tissue. This example demonstrated the model's ability to accurately detect and classify malignant cases, which is critical for early diagnosis and clinical decision-making.

All these outputs are indicators of the model being able to classify as well as provide high-confidence estimates, both of which are essential in clinical acceptance and decision-making processes. The gradients in intensity are smooth, and the tumor localities are well-defined, which helps the attention mechanisms to target the discriminating areas successfully.

Conclusion and Future Work

This research paper proposed a ViT-based methodology for automatic classification of lung cancer on CT image data. Figure 9 represents a representative chest CT slice classified by the model as a benign case with a predicted confidence of 99.83%. The image shows well-defined lung structures without irregular or invasive features typically associated with malignancy.

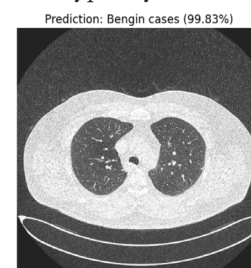


Figure 9: The output of the model prediction for a CT scan image classified as a benign case has a confidence score of 99.83%. The image highlights the model's ability to identify non-malignant features within the lung region, indicating high confidence in distinguishing benign abnormalities from other classes.

The model predicted the CT scan as a benign case with very high confidence (99.83%), indicating strong reliability in its classification. The lung region showed localized and well-defined structures, without the aggressive, irregular masses typically seen in malignant cases. The tissue appearance is relatively uniform and less heterogeneous, suggesting non-cancerous characteristics. There were no clear signs of widespread abnormal growth or invasion, which supports a benign interpretation. The high confidence score indicated that the model successfully identifies subtle features that distinguish benign from malignant and normal cases. Overall, this example demonstrated the model's ability to accurately recognize benign conditions, which is important for avoiding unnecessary clinical intervention.

Exploiting the mechanism of self-attention that transformer architectures possess, the proposed model was more effective in learning global contextual dependencies among pulmonary structures, which can be ineffective with conventional convolutional models. To promote the reliability and transparency of the diagnostics, the pipeline combined advanced preprocessing, patch-wise tokenization, and interpretability.

Wide-scale experiments on a labeled CT scan dataset also showed that the proposed model was more effective than baseline CNNs and recent models based on transformers in a variety of evaluation metrics. It was found to have a macro-average accuracy of 92%, a macro-average F1-score of 83%, and an AUC of 98%, which is considered to have good generalization between benign, malignant, and normal. The confusion matrix and ROC analysis also confirmed that the model can discriminate between diagnostically similar cases, particularly between benign and normal CT presentations.

The model also performed well on unseen samples, generating predictions with high confidence, along with quantitative benchmarks, which is another positive sign that the model can be utilized in the real-world setting. The Ablation studies were able to demonstrate the importance of patch size and transformer depth in the performance of classification, which can be used practically to renovate the design in the future.

From a clinical implementation standpoint, the proposed model shows promise as a decision-support tool that could assist radiologists in triaging CT scans, particularly for flagging high-confidence malignant cases for priority review. The high AUC (0.98) and strong malignant-class recall suggest the model could meaningfully reduce missed detections in screening workflows. However, deployment in real clinical environments would require prospective validation on multi-institutional datasets, integration with existing PACS infrastructure, and regulatory review. The model's attention maps offer a preliminary basis for explainability, though further work is needed to correlate attention activations with established radiological findings before clinical adoption can be recommended. The modern approach has its limitations, even though it has certain advantages. The model was based on a mid-sized, class-imbalanced dataset, which can affect the performance of underrepresented groups of cases, such as benign ones. Besides, although the attention maps do provide some interpretability, additional studies are necessary to align the at-

tention maps with domain-specific radiological characteristics to build clinical trust.

The research would be improved in the future by focusing on these limitations in a variety of ways. To start with, we aimed to include multimodal data, such as histopathological images and patient metadata, to add to the context of diagnosis. Second, a self-supervised and semi-supervised learning paradigm could be used to mitigate the reliance on labeled data. Third, we studied the variations of lightweight transformers and model compression methods that are hardware-sensitive to enable implementation in the resource-limited clinical setting. Lastly, cross-institutional datasets were also sought to support clinical validation to evaluate generalizability and strength in different populations.

To summarize, the presented work contributed to a new, interpretable, and high-performing ViT-based lung cancer classification solution, which will provide a foundation for a scalable, AI-assisted diagnostic aid in thoracic cancer cases.

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Development of Peptide-based Nanotubes with Increased Resistance to Enzyme Degradation

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ABSTRACT: This systematic literature review evaluates strategies for enhancing proteolytic resistance in peptide-based nanotubes (PBNTs), self-assembling cylindrical nanostructures with therapeutic potential in drug delivery applications in medicinal treatments such as drug delivery. The primary focus is on chemical and structural modifications of drug molecules that improve PBNT stability in vivo, along with discussion on the mechanisms, limitations, strengths, and implications of each strategy, as well as future therapeutic applications. The review also highlights the need to integrate multiple strategies to achieve different functionalities needed for a specific drug application. Further research should include in vivo studies and computational modeling via machine learning to validate these strategies and refine PBNT performance. This review additionally emphasizes the critical need for developing PBNT carriers that are both durable and functionally versatile for the field of medical therapeutics. This article is based on qualitative research utilizing peer-reviewed articles, journals, and various studies from the 2000s.

KEYWORDS: Biochemistry, Medical Biochemistry, Peptides, Degradation Enzymes Resistance, Nanotubes.

■ Introduction

The field of drug delivery focuses on how therapeutic compounds can make their way throughout the body. Despite significant advances in pharmaceutical sciences, several limitations persist in contemporary drug delivery systems. These include the lack of precise targeting, poor bioavailability, cytotoxicity, and complicated administration, which can limit the efficacy of the drug.¹ Recently, nanotechnology has shown promise as a tool to develop more precise and efficient drugs. In particular, peptide-based nanotechnology has become an emerging topic in the field of drug delivery. Due to their unique properties, including enhanced bio-barrier permeability, self-assembly, and precision-guided delivery, it holds strong promise for future medical applications.¹⁻³

In recent years, researchers have focused on designing nanostructures that resemble biological molecules and can serve similar functions. Peptides are widely viewed as a viable solution for this. Their versatility and amino acid building blocks allow for tunable chemistry. This modular chemistry allows researchers to choose how they interact with various cells in the body.⁴

Peptides are polypeptide chains of 50 or fewer amino acids. They have shown tremendous potential to treat diseases such as cancer, cardiovascular disease, and diabetes, but many challenges still exist: low oral bioavailability, short half-lives, and enzymatic susceptibility.¹² Structurally, peptides are chains of amino acids with an amino group called the N-terminal at one end, and a carboxyl group called the C-terminal at the other. Amino acids grow by forming a peptide bond between the C-terminal and the amino group of a free amino acid.¹³ Peptides can then be gathered into different types of nanostructures, such as hydrogels, nanotapes, nanonecklaces, nanofibers, as shown in Figure 1.¹⁴ Peptide-based nanotubes

are composed of cyclic, disk-shaped peptides and have shown the ability to self-assemble under certain conditions.^{6,19}

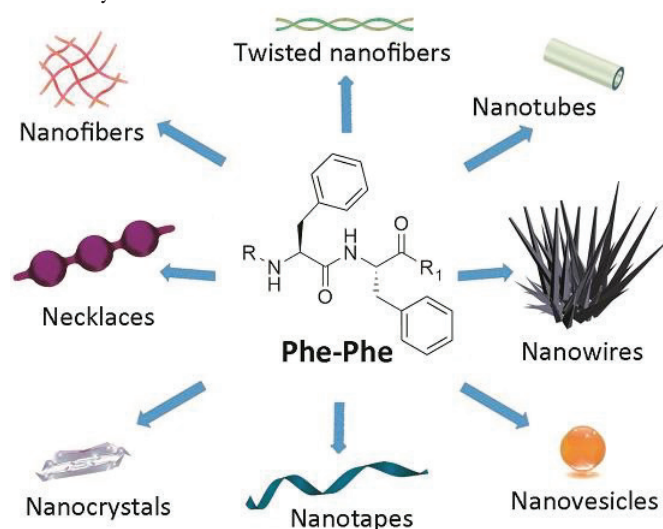


Figure 1: Graphical representation of the different types of nanostructures.⁴¹

The formation of peptide nanotubes can come from molecular self-assembly that allows the peptide to align in specific organizations, reducing system free energy and increasing molecular stability. Hydrogen bonding is one of the key forces to drive self-assembly. For PBNTs specifically, they create a path for tubular assembly. A cluster of peptide molecules is chosen as a “seed,” and others branch off from the main sequence, making the process highly sequence dependent. Changes in pH, temperature, and solvent polarity can all influence the kinetics of peptide self-assembly. It has the potential to provide a modular framework for designing adaptive biomaterials in medicine.¹⁶

Despite all the advantages of peptide nanotechnology, one of their most widely recognized challenges is their susceptibility

to enzymatic degradation by proteases.⁵ However, a specific type of peptide nanoparticle, called a peptide-based nanotube (PBNT), could be used with different strategies to increase proteolytic resistance.^{5,6} PBNTs are well-defined hollow cylinders with a diameter range of 0.5–500 nm and an aspect ratio greater than 5.⁶ This type of structure makes them easily modifiable, which can be used to increase proteolytic resistance.⁶ While various strategies like cyclization, side-chain modification, and conjugation have been explored in peptides, not many studies have focused narrowly on PBNTs.⁵ This highlights a critical gap for research in PBNTs that can safely and effectively circulate throughout the body.⁶

From a chemical perspective, peptides should take several hundred years to fully hydrolyze on their own. However, the degradation of peptides *in vivo* occurs when Proteolytic enzymes recognize specific amino acid sequences and catalyze the hydrolysis of peptide bonds, resulting in peptide fragmentation and loss of biological activity. The rate at which this degradation occurs is affected by factors such as the conformation of the peptide, the amino acid sequence, and the accessibility of the bonds to the protease. In summary, the degradation of peptides and PBNTs is facilitated by proteases acting as a catalyst in the hydrolysis of peptide bonds.⁷

Addressing this weakness in PBNTs can lead to a variety of benefits, such as antiviral effects, gene delivery, and improved drug delivery.⁸ Specific PBNTs are able to inhibit viral replication. This mechanism may enable the inhibition of influenza A virus replication and other viral diseases.⁹ Moreover, their unique tubular structure allows the inner section to hold drugs while the outside can be modified or functionalized for targeting specific receptors or shielding from enzymes.⁴ Other types of PBNTs have been proven to reduce harmful activity in a corneal injury model via gene therapy and even have the potential to be ingested orally and carry DNA to other tissues in the body.¹⁰ Research also demonstrates that PBNTs effectively deliver anticancer drugs with controlled release due to their hollow nature.¹¹ Developing strategies to protect nanotubes from proteolytic breakdown would expand their use across many diverse fields of medicine, including but not limited to: immunotherapy, regenerative therapy, and controlled release systems.

Beyond direct medical applications, PBNTs also offer a new way of thinking about modern medicine. They offer a more modular approach, allowing researchers to fine-tune drugs from the top down. This approach could offer doctors and scientists more control over how their medicine would act *in vivo*. Therefore, further research work is warranted to expand the creation of optimized PBNTs for various physiological conditions. This research study will recommend methods to fully utilize the potential of PBNTs by increasing their proteolytic resistance within the body. By examining strategies used to modify other types of peptides, this paper will compare and apply their methods to nanotubes instead, evaluating which modifications will be the best suited for maintaining structural integrity *in vivo*. No lab work or clinical trials will be done. Instead, this paper will use hypothetical and logical explanations based on existing knowledge of peptide chemistry and

nanostructure design. This review narrows down the topic of peptides to only PBNTs due to their superior proteolytic resistance, aiming to provide a framework for future experimental investigations that could translate these strategies into practical applications.

■ Methodology

This work is a literature review, not an experimental investigation. All data were derived from secondary, peer-reviewed sources, rather than laboratory experimentation. The purpose of this approach was to evaluate existing findings rather than to find new data. A comprehensive review method was used to effectively provide a complete understanding of current advancements, limitations, and potential directions for PBNT use in the future, as it relates to biomedical contexts.

To conduct this review, a literature search was carried out using databases like PubMed and Google Scholar. Additional databases such as ScienceDirect and Scopus were also included to ensure coverage of nanotechnology and biomedical publications. The temporal scope was restricted to publications from 2015 to 2025 (except cited refs. 6 and 13) to ensure current relevance. Search terms including 'peptide nanotubes,' 'drug delivery,'... were combined using Boolean operators to gather information on the nanotubes, peptides, applications, and ways to increase proteolytic resistance. This data on peptides was applied to PBNTs to see how similar methods could be used to increase their proteolytic resistance.

The initial searches came up to around 5000 papers in total. However, after removing duplicates and scanning through titles and abstracts for relevance, a final set of 20 studies was selected for an in-depth review. Papers that didn't mention or reference PBNTs weren't included. Further papers were manually included to provide context or collected from papers already found.

Inclusion criteria included addressing PBNT or related peptide nanostructures, addressing biomedical or therapeutic applications, and being peer-reviewed in English. Papers that were excluded were lacking primary data, specificity to PBNTs, or clarity. Only original research articles, literary reviews, and studies with clear and methodical reporting were included. Studies without a clear context were removed to maintain academic rigor.

No physical instruments, materials, or laboratory techniques were used. As this study relied on publicly available academic publications, no ethical approval or considerations were necessary.

Because this study did not involve human or animal subjects, ethical review was not necessary. However, academic integrity protocols were strictly followed, including accurate citations, avoidance of plagiarism, and evaluation of each source's credibility and quality.

Analysis highlighted several techniques to increase enzymatic resistance within PBNTs, such as cyclization, D-amino substitution, and chemical capping. Furthermore, the research revealed that PBNTs showed great promise in therapeutic applications. Beyond highlighting existing research and identifying trends, this review aimed to synthesize the findings into

a conceptual and easy-to-understand guide for future peptide design and study.

A limitation of this research is that it is mostly hypothetical. There is a lack of existing laboratory research focusing on increasing proteolytic resistance in PBNTs or even peptides in general. Most current studies focus on structural formations or early-stage degradation. Few explore long-term behavior or effects *in vivo*.

However, by identifying clear mechanisms to increase proteolytic resistance in PBNTs, this paper could provide a foundation for future work for improved PBNTs.

■ Structural Designs and Proteolytic Resistance Strategies

The characteristics of PBNTs come from the sequence, chemical composition, and modifications of their peptide building blocks. They dictate their self-assembly, morphology, and functional potential. Self-assembling peptides derive from single amino acids, dipeptides, cyclic peptides, D/L peptides, and functional or lipidated variants. They provide tunable structural characteristics.^{8,17,18} Cyclic peptides form PBNTs by stacking on top of each other, with amino and carbonyl groups faced perpendicular to the ring, stabilizing the structure through hydrogen bonding between the oxygen in the carbonyl group and the nitrogen in the amino group. When alternating D- and L-type amino acids, the symmetry allows them to stack neatly and form a PBNT.¹⁹ Dipeptides, a peptide made from 2 amino acids, are the shortest and simplest form of peptides. To self-assemble, they need 2 hydrophobic amino acid residues and hydrogen bonding. Intermolecular forces, such as π - π stacking, Van Der Waals forces, and hydrogen bonding, let dipeptides form a nanotube shape.⁴ Lipidated peptides, also known as lipopeptides, are hydrophobic oligopeptides with attached lipid chains that make the whole molecule amphiphilic.²⁰ A study by Castelletto *et al.* showed how a lipopeptide (C16KTT β AH) could self-assemble in water due to its lipid tail and potentially have selective anti-cancer effects.²¹ Single amino acids could be modified and self-assembled for drug delivery or other therapeutic applications such as cell culture, photocatalysis, and antibacterial applications. They can also possess fast self-assembly kinetics and excellent physical and chemical properties; however, the peptide may lose aspects of its biological function, such as the ability to form metal complexes or its antimicrobial properties.^{22,23}

Key strategies for controlling PBNT structure include taking advantage of cyclization and heterochirality. Cyclic peptides form β -sheet-like tubular structures, with the peptide backbone inside the tube and side chains exposed on the outside. This lets us control nanotube diameter, surface chemistry, and rigidity, while increasing proteolytic resistance.^{17,18,24} Cyclization is one of the most promising strategies. It constrains the peptide backbone, reduces flexibility, and limits enzymatic access points. Empirical evidence demonstrates that conformationally constrained peptides exhibit enhanced receptor binding affinity and enzymatic resistance.¹⁷ Peptide nanotubes are formed from stacking amino acid monomers. Linear monomers could be used, but they have less proteolytic

resistance compared to cyclized monomers. Through cyclization and the exploitation of heterochiral amino acid properties of amino acids, alternating types of amino acids could be used to form a PBNT with more rigidity and enzymatic resistance than one composed of linear monomers.⁶ Cyclization comes in four forms: head-to-tail, head-to-side chain, side chain-to-side chain, and tail-to-side chain. Head-to-tail cyclization leads to rigid peptide structures and predictable assembly, while also blocking off free N- and C- termini that proteases attach to, increasing proteolytic resistance.²⁵ Furthermore, a study by Clover *et al.* showed that by substituting D-amino acids for L-amino acids, it could influence the molecular-level packing and the supramolecular morphology, increasing rigidity and the location of termini, potentially increasing proteolytic resistance.²⁶ However, it could potentially lose its ability to bind to metal receptors or metal binding affinity.²⁷

Chemical modifications like capping the N-terminal, halogenation, and side-chain analogs can enhance π - π stacking, making the PBNT more rigid.^{3,4,18} While also stabilizing the structure, they can increase functionality by adding more sites for functionalization. Separate studies by Vernon and Wener showed that capping aromatic groups on the N- and C- termini of proteins led to more π - π stacking, which could increase rigidity and make it harder for proteases to attach to and cleave backbone bonds.^{28,29} Halogenation is the process of introducing halogens into a chemical compound. When used in peptides, they have the potential to increase proteolytic resistance; however, they cannot be generalized. It depends on the substitution position and the number of halogens introduced.³⁰ Furthermore, a study by Glibowicka *et al.* showed that Lys-chain shortening could protect against enzymatic degradation. By making the terminal amine side chain shorter and less flexible, the target site could become hidden and less accessible to the protease, increasing proteolytic resistance.²⁴

To further extend stability, other types of backbone modifications could be utilized. N-terminal acetylation, C-terminal amidation, and incorporation of D-amino acids could increase enzymatic resistance.³¹ It would be especially useful for PBNTs, where exposed terminal points could let the protease interact with them.

N-terminal acetylation is where an acetyl group ($-\text{COCH}_3$) is added to the first amino acid of a peptide or a protein, the α -amino group.³² If PBNTs are Nt-acylated, they could potentially resist enzymatic cleavage or degradation, because they could block flagging for degradation from E3 Ub ligases.³³ Moreover, a study by Kang *et al.* showed that Nt-acetylation prevented enzymatic degradation.³⁴ C-terminal amidation is replacing the free carboxyl group ($-\text{COOH}$) at the C-terminal end of the peptide with an amide group ($-\text{CONH}_2$). This has been shown to enhance structural stability and antimicrobial activity.^{34,35} On the other hand, D-amino acids are enantiomers of more commonly occurring L-amino acids. Given the stereoselectivity of human and microbial proteases for L-amino acids, substituting them with D-amino acids in PBNTs could lead to enhanced peptide stability against protease degradation.³⁴

Additionally, encapsulation could be used to create a protective barrier around bioactive peptides. Literature indicates liposomal encapsulation as an optimal strategy because of its bioavailability, non-toxicity, and amphipathic characteristics.³⁶ Since liposomes are also lipids, they are biocompatible and thus considered safe by the Food and Drug Administration (FDA).^{37,38} Liposomes also promote targeting of diseased cells.³⁸

A summary of the stabilization strategies for PBNTs and their relative advantages and limitations is shown in Table 1. Overall, each strategy aims to enhance stability, but comes with its respective advantages and limitations, implying that the purpose of the peptide must be considered when choosing a strategy.

Table 1: Summary of stabilization strategies for peptide-based nanotubes (PBNTs) and their relative advantages and limitations.

Strategy	Mechanism	Advantages	Limitations
Cyclization	Constrains the peptide backbone	Enhances rigidity and proteolytic resistance	Could hinder self-assembly or biological activity in PBNTs
D-amino incorporation	Alternating D- and L-amino acids improves symmetry and stacking	Increases packing and provides proteolytic resistance	Could hinder functional activity such as metal binding
N-terminal acetylation	Adds acetyl group on α -amino group	Enhances proteolytic resistance	Could affect functional impact
C-terminal amidation	Replaces -COOH group with -CONH ₂	Enhances proteolytic resistance	Could affect functional impact
Halogenation	Adds halogen atoms onto the peptide chain	Could increase rigidity and proteolytic resistance	Effects not easily predictable; not enough research
Aromatic capping	Caps aromatic groups onto N- and C- termini of proteins	Enhances π - π stacking, improves rigidity	Can alter self assembly into nanotubes
Lipidation	Attach lipid chains onto peptide	Increase self-assembly properties and possibly give anti-cancer effects	Could reduce solubility and cause aggregation
Single amino acid modifications	Modify individual residues	Allows for fine control	Impacts may be hard to predict
Encapsulation	Surrounds peptides with carriers like liposomes	Improves stability, bioavailability, and targeted delivery	May impact release rate

■ Therapeutic Applications of PBNTs

Drug delivery is one of the most promising applications of PBNTs. Their hollow cylindrical structure lets small molecule drugs be efficiently encapsulated, and the modifiable peptide backbone allows for chemical functionalization, which can add many helpful properties, such as controlled release, targeting specificity, and biocompatibility. Studies suggest therapeutic precision while minimizing potential harmful side effects.^{3,4}

Studies show that PBNTs can deliver anticancer drugs with enhanced selectivity and reduced off-target toxicity. Since the proteins on tumor cells often differ from those of normal cells, using strategies like phage display could be used to find those specific proteins and molecular markers. Once they are found, specific PBNTs carrying cytotoxins could bind to the surface of the affected cell. This suggests an increase in the efficacy of the drug and a reduction of unwanted effects on normal cells.³⁹

Functionalized nanotubes could be used to cross biological barriers and carry DNA or RNA cargos. This demonstrates potential in localized and systemic administration. Because of their interchangeable surface chemistry, PBNTs could be used to selectively target nucleic acids, which may protect them from degradation and enable targeted release within cells.

These studies suggest PBNTs can protect the DNA from acid, bile, and enzymes, and improve permeability and stability, letting it reach many different tissues.⁸

Studies show that some PBNTs can interfere with the pathway to viral reproduction. They can block viral entry or replication machinery.³¹ This suggests that PBNTs could be used for broad antiviral therapy. A study by Montero *et al.*⁴⁰ discovered cyclic peptides with anti-hepatitis C virus (HCV) activity. The specific characteristics needed for anti-HCV activity include chemical and structural modifications, a cyclic backbone configuration, and a pattern of basic and acidic amino acids in the peptide sequence.⁴⁰

Self-assembling peptides in corneal injury models have been shown to reduce harmful activity through controlled gene expression and bioactive delivery. This is especially significant since the epithelial layer, which restricts molecular entry in normal drugs, has been bypassed. This implies that some PBNTs with similar structures could distribute to the duodenum, liver, stomach, and kidneys.¹⁰

Finally, to address the growing problem of antibiotic resistance in bacteria, cyclic peptides could offer a promising alternative. Peptides with amphiphilic properties, meaning they have both hydrophilic and hydrophobic regions, are particularly effective. In fact, studies have shown that even a single substitution of an amino acid with one containing hydrophilic elements can significantly enhance antibacterial activity. This modification has been effective against several bacterial strains, including MRSA, *E. coli*, *U. linza*, and *N. perminuta*, demonstrating that small changes in peptide composition can have a substantial impact on antimicrobial properties and suggesting that cyclic peptides could be further optimized as novel antibacterial agents.⁸ This suggests PBNTs, which are very similar to cyclic peptides structurally, could have the same effects.

■ Discussion

This review explored ways to enhance enzyme resistance in PBNTs and lists various viable methods. Cyclization, β -sheet cyclization, and terminal protection are recognized as means to achieve increased PBNT stability. Chemical modifications such as side-chain modifications and the addition of noncanonical amino acids further reduce enzymatic degradation and improve the half-life of these nanotubes in biological environments. For treatment, PBNTs work well for drug and gene delivery, antiviral and regenerative applications, and self-assembly. Beyond the main results, a key takeaway is how closely tied these factors are. Minor molecular modifications, such as D-amino incorporation or N-terminal acetylation, can drastically affect the chemical properties of the PBNT. Thus, each sequence of the PBNT must be precisely aligned with the desired functional outcome of the drug.

Furthermore, the findings of this study suggest that proteolytic resistance strategies could be highly dependent on the environment in which the drug is used. Different types of treatments would each require specific modifications of the PBNT. In some ways, this could be considered a strength of PBNTs, seeing how they can be readily adaptable to many different scenarios.

Through review of the limitations and strengths of the methods for increasing proteolytic resistance, Cyclization represents the optimal strategy for PBNT stabilization, as it simultaneously enhances proteolytic resistance through backbone constraint while maintaining favorable self-assembly characteristics. D-amino substitution, N-terminal acetylation, and C-terminal amidation also stand out as notable methods to increase enzymatic resistance. D-amino substitution and cyclization could be combined to develop PBNTs with increased resistance and self-assembly properties, although more research is needed to ensure no adverse effect on functional ability. Finally, N- and C- terminal modifications could lead to an increase in enzyme resistance as well as functional activity.

The implications are that PBNTs could become a useful, customizable, and versatile tool in therapeutics, especially if research focuses on the *in vivo* stability of PBNTs. However, more experimental work that focuses on computational modeling and laboratory experiments would be needed for PBNTs to be used in medicine.

■ Conclusion

This review article discusses the potential benefits and drawbacks of different drug molecule modification strategies and highlights the need for adopting the integration of multiple strategies to meet specific drug therapeutic applications. Considering all the various factors, the author concludes that cyclization may serve as a foundational design strategy for peptide-based nanotubes (PBNTs), yielding highly predictable structural dynamics conducive to machine learning integration for forecasting stability and efficiency. Furthermore, the modular architecture of cyclized PBNTs facilitates the conjugation of functional moieties, enabling the development of sophisticated, stimuli-responsive systems with expanded therapeutic potential.

In summary, advancing PBNT design through chemical and structural means could overcome one of the main barriers in peptide-based drug delivery and bring emerging nanotechnology into therapeutics.

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Short-Term Effects of Musical Participation on Adolescents' Self-Efficacy: A Pretest–Posttest Study

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ABSTRACT: This study examines the effect of musical participation on adolescents' self-efficacy using a one-shot pretest–posttest design. A total of 30 high school students voluntarily took part in a structured musical activity, which involved singing a self-selected musical or film OST piece. The General Self-Efficacy Scale (GSES), including 10 standard items and 2 custom items related to stage confidence and emotional expression, was administered before and after the activity. Data were analyzed using reliability testing, paired-sample t-tests, and Pearson correlation. The results revealed a statistically significant increase in self-efficacy scores following musical participation ($t = -4.58, p < .001$). The greatest improvements were observed in items related to stage confidence, stress regulation, and multitasking. Although not statistically significant, a negative correlation was observed between initial self-efficacy and score improvement ($r = -0.35, p = 0.057$), suggesting that students with lower initial confidence may benefit more from the intervention. These findings suggest that short-term musical engagement can serve as a meaningful psychological boost for adolescents, particularly those with lower baseline self-efficacy. The study highlights the potential of school-based musical activities as effective tools for promoting emotional regulation and building confidence during adolescence.

KEYWORDS: Behavioral and Social Sciences, Sociology and Social Psychology, Adolescent Mental Health, Self-Efficacy Assessment, General Self-Efficacy Scale (GSES).

■ Introduction

Research Motivation:

Adolescence is a critical period during which individuals shape their sense of direction and identity. Self-efficacy formed at this stage significantly influences future decisions in areas such as career choices and interpersonal relationships. However, today's adolescents often experience self-doubt, a fear of failure, and a feeling of helplessness, driven by intense academic competition and the pressures of social media. These observations prompted us to explore potential methods for enhancing adolescent self-efficacy.

As the president and vice president of the musical theater club at the Korean Minjok Leadership Academy, the authors have extensive experience planning performances, coaching peers, and engaging in vocal and dance rehearsals. Throughout this process, we frequently observed that students found relief from stress and gained a sense of joy and accomplishment through musical practice. The collaborative experience of memorizing lines, expressing emotions, singing, and performing as a group seemed to transcend the typical boundaries of extracurricular activities. These experiences inspired the central question of this study: Can musical participation serve as more than a leisure activity and contribute meaningfully to the psychological development of adolescents? To address this, we aimed to systematically and quantitatively examine the impact of musical engagement on adolescent self-efficacy.

Literature Review:

1) Self-Efficacy:

Self-efficacy refers to an individual's belief in their capacity to execute specific behaviors necessary to produce a desired outcome.¹ Bandura defined self-efficacy beliefs as a crucial determinant of human motivation, emotion, and behavior, exerting influence through motivational, cognitive, and affective mediators.² These beliefs enable individuals to regulate their behavior and respond proactively to environmental demands.

According to Bandura, self-efficacy is developed through four primary sources of information,² as illustrated in Figure 1:

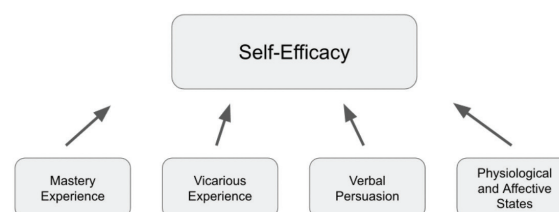


Figure 1: Four Sources of Self-Efficacy as Proposed by Bandura: Mastery Experience, Vicarious Experience, Verbal Persuasion, and Physiological & Affective States. This framework explains how mastery, modeling, encouragement, and affective cues shape efficacy beliefs; in this study, musical rehearsal naturally engages all four sources.

- **Mastery Experience:** Individuals tend to engage in tasks they believe they can accomplish and avoid those perceived as too difficult. Bandura posited that repeated success in past tasks builds high self-efficacy, while repeated failure lowers it.
- **Vicarious Experience:** Observing others, particularly peers or role models, succeed in tasks can lead individ-

uals to believe they can grow as well, and the reverse is also true.

- Verbal Persuasion: Encouragement or discouragement from others—such as praise or criticism—can strongly influence an individual's belief in their capability.
- Physiological and Affective States: Physical and emotional conditions can influence how individuals judge their strengths and weaknesses.³

These sources collectively shape how a person evaluates their capabilities, forming a psychological construct shaped through interactive experiences and internal states.

In addition to its theoretical underpinnings, self-efficacy possesses unique attributes that distinguish it from related concepts such as self-esteem and confidence.⁴ These include:

- Judgment of Capabilities: Rather than focusing on innate traits, self-efficacy centers on one's perceived ability to accomplish a specific task.
- Multidimensionality: Self-efficacy is not a singular trait—it can vary across different domains such as mathematics, language, or the arts.
- Context-Dependency: Beliefs about one's abilities can fluctuate depending on the situation. For example, competitive environments may reduce self-efficacy, whereas collaborative contexts may enhance it.
- Mastery-Based Assessment: Self-efficacy is evaluated based on whether one believes a task can be accomplished, rather than through comparison with others.
- Prospective Nature: Self-efficacy is measured before action takes place, making it a predictive factor rather than a *post hoc* outcome.

Various empirical studies also confirm the practical implications of self-efficacy. For instance, higher self-efficacy has been linked to greater interpreting accuracy among language learners and to stronger job competence among paramedics.^{5,6}

2) Adolescents and Self-Efficacy:

Adolescence, a transitional stage between childhood and adulthood, typically includes individuals aged 10–18 in South Korea, though definitions vary globally. Erikson described the core developmental task of adolescence as the establishment of identity, which requires a “moratorium” period in which individuals can integrate previously developed components of the self.⁷ Failing to do so may lead to identity confusion, expressed through frustration or negative behavior.

Bandura emphasized that goal-directed behavior is sustained when individuals believe in their capacity to succeed.² Thus, self-efficacy plays a foundational role in shaping adolescents' perception of their identity and their chosen roles in society.

Puberty brings rapid cognitive, emotional, and social changes. Adolescents become more sensitive to external evaluations, particularly from teachers and peers, and self-efficacy becomes closely tied to their social environment. Consequently, self-efficacy is a crucial factor in adolescent development and the formation of identity.

3) Arts Participation and Self-Efficacy:

The Korean Standard Dictionary defines “art” as all human activities and products aimed at expressing and creating beauty. Previous research has indicated that various forms of artistic engagement can positively affect adolescents' psychological development. For example, Seong and Kim found that participation in youth orchestra programs significantly improved general self-efficacy ($p < .001$).⁸ Hughes and Wilson also demonstrated that youth theatre promotes personal and social development among adolescents.⁹ Additionally, Jo and Koo reported significant improvements in self-esteem, creativity, and appreciation for the arts based on adolescents' hours of participation in cultural and artistic programs.¹⁰

4) Musical Theatre and Self-Efficacy:

Musical theatre is a modern American art form that combines music, singing, and dance, often incorporating elements of revue, spectacle, and showmanship. According to the National Institute of the Korean Language, it is defined as a comprehensive stage art that integrates musical comedy, musical plays, and other performative elements.

Musical participation inherently encompasses various sources of self-efficacy, including mastery experience (such as memorizing lines and rehearsing choreography), vicarious experience (observing and comparing oneself to peers), and verbal persuasion (receiving applause from audiences). Prior research suggests that engaging in musical activities can improve emotional regulation in middle school students.¹¹

Taken together, these studies suggest that artistic experiences can enhance self-efficacy and personal growth. Given that musicals integrate multiple forms of art—including theatre and music—it is hypothesized that musical theatre may have an especially strong effect on self-efficacy. Therefore, the present study aims to empirically test the impact of musical theatre participation on adolescents' self-efficacy.

Conceptual Framework and Hypotheses:

1) Research Model:

This study aims to empirically examine the effect of musical rehearsal participation on changes in adolescents' self-efficacy. By measuring self-efficacy scores before and after participation in musical activities, the study examines whether such experiences lead to statistically significant improvements.

- Independent Variable: Participation in musical rehearsals (pre/post comparison)
- Dependent Variable: Change in self-efficacy scores (measured as score difference)

The research model is illustrated in Figure 2.



Figure 2: Research Model: The Effect of Musical Rehearsal Experience on Changes in Self-Efficacy. The single-group pre–post design tests whether rehearsal elevates self-efficacy; subsequent analyses supported the model, showing a significant mean increase after participation.

A single-group pre-post design is used, and a paired t-test is conducted to compare mean differences in self-efficacy before and after participation. Additionally, item-level changes and correlations between baseline scores and the magnitude of change are analyzed to enable a more detailed interpretation.

2) Research Questions and Hypotheses:

The study addresses the following research questions:

- How does participation in musical theatre affect adolescents' self-efficacy?
- What correlations exist among individual items and subfactors of self-efficacy?
- Does the effect of participation vary depending on the participant's initial self-efficacy level?

Based on these questions, the following hypotheses are proposed:

- H1: Adolescents will show a statistically significant increase in self-efficacy scores after participating in musical rehearsal and performance.
- H2: There will be a significant positive correlation between GSES items and supplementary survey items reflecting musical participation.
- H3: Participants with lower baseline self-efficacy will show greater improvements after participation.

■ Methods

Research Design and Procedure:

This study employed a one-shot pretest-posttest experimental design to examine the effect of musical participation on adolescents' self-efficacy. The experiment was conducted in June 2025 in an experimental space set up on the 2nd floor of Deokgo Hall at the Korean Minjok Leadership Academy (KMLA).

Before the experiment, the researcher coordinated schedules with participants and allocated approximately 6 minutes of individual session time per participant. To ensure psychological stability and immersion in the task, each participant was asked to submit in advance a link or instrumental (MR) file of a musical or movie OST they were familiar with and wished to perform. This approach aimed to reduce tension and enhance natural emotional expression and self-awareness by avoiding unfamiliar songs.

Upon arrival at the experiment site, participants were first asked to complete a pretest using the General Self-Efficacy Scale (GSES). The questionnaire consisted of 12 items: 10 standard items from the GSES and 2 additional items specifically developed by the researcher to assess confidence and emotional expression in musical contexts. These two items were included in both the pretest and posttest to ensure consistency and measure psychological responses in performance settings.

After completing the pretest, the researcher left the room, allowing the participant to sing freely along with their pre-submitted MR. Participants were permitted to view lyrics during the performance, and researchers remained outside, only monitoring the audio playback without interfering in any way. This

arrangement was designed to help participants feel secure and express themselves authentically.

Immediately after the performance, the researcher re-entered the room and administered the posttest using the same 12-item questionnaire. The response format and environment were kept identical to those of the pretest to enable consistent comparisons.

Songs used in the experiment included familiar musical and Disney OSTs such as "A Whole New World," "Do You Hear the People Sing?," "How Far I'll Go," "This is Me," and "Let It Go." Participants were free to choose songs that reflected their identity or aspirations, encouraging natural emotional flow through self-expression, immersion, and self-awareness.

Participants:

This procedure was consistently applied to all 30 participants. The researchers minimized intervention throughout to ensure the internal validity of naturally occurring changes in self-efficacy.

This study was conducted in June 2025 with 30 first-year students enrolled at the Korean Minjok Leadership Academy (KMLA). Participants were recruited based on their interest in musical activities and their willingness to participate voluntarily in the study.

On the day of the experiment, participants confirmed their consent by checking a statement at the beginning of the questionnaire: "I agree with the purpose of the study and voluntarily participate." All participants were required to check this item to proceed with the survey, thereby ensuring the ethical validity of the survey.

The 30 participants were divided into three cohorts and took part in the experiment sequentially. The number of students and gender distribution for each cohort are shown in Table 1:

Table 1: Participants' Grade and Gender Information. Thirty adolescents (16 male, 14 female; ages 15–17) participated, providing a balanced sample for pre-post comparisons.

Age	Total	Male	Female
17	14	9	5
16	10	4	6
15	6	3	3

Of the 30 participants, 16 were male, and 14 were female, resulting in a relatively balanced gender ratio. Since the study focused on adolescents, the experimental environment was designed to minimize psychological burden and external evaluation. Participants were explicitly informed that the experiment did not involve evaluating or comparing singing ability. They were also reminded of their right to withdraw from the experiment at any time. The setting was carefully arranged to ensure psychological comfort and allow for authentic self-expression without external interference.

Instruments:

1) General Self-Efficacy Scale (GSES):

To measure changes in self-efficacy before and after musical participation, this study employed the General Self-Efficacy Scale (GSES). Originally developed by Schwarzer and Jerusalem based on Bandura's self-efficacy theory, the GSES assesses

an individual's belief in their ability to solve problems and achieve goals, even in difficult situations. The Korean version of the scale was used for this study.

The original GSES consists of 10 items, each rated on a 5-point Likert scale (1 = Not at all true to 5 = Exactly accurate). Higher scores indicate higher general self-efficacy.

In addition to the original 10 items, the present study included two researcher-designed items specifically tailored to assess self-expression in musical contexts. These items were added to more precisely measure confidence in stage performance and psychological responses in expressive settings. Thus, the final survey consisted of 12 items and was administered both before and after the musical activity, with the item order and conditions held constant to ensure valid comparisons.

The exact questionnaire is provided in the Appendix. The surveys were administered via Google Forms. Participants completed the pretest in writing upon arrival at the experiment site and responded to the posttest in the same manner after the musical performance. The wording and order of the pre- and post-surveys were identical to ensure consistency.

2) Reliability Analysis:

Table 2: Cronbach's α Reliability Coefficients for Pre- and Posttest Surveys. Internal consistency was high and improved from pretest ($\alpha = .858$) to posttest ($\alpha = .908$), supporting the reliability of the 12-item instrument.

Type	No. of Items	Cronbach's α
Pretest	12	0.858
Posttest	12	0.908

The study employed pre- and post-surveys to assess the impact of musical participation on self-efficacy. As shown in Table 2, all 30 students completed both surveys, which included the same 12 items at each time point.

To assess internal consistency, Cronbach's α was calculated. For all 12 items, the reliability was $\alpha = 0.858$ for the pretest and $\alpha = 0.908$ for the posttest, indicating high internal consistency of the instrument.

Additionally, when excluding the two musical context-specific items (Item 11: "I am confident singing or acting on stage" and Item 12: "I feel that I can naturally express my emotions"), the α values improved: $\alpha = 0.890$ for the pretest and $\alpha = 0.916$ for the posttest. This suggests that the two additional items may measure slightly different constructs from general self-efficacy.

These results support the reliability of the instrument used and its appropriateness for analyzing changes in self-efficacy before and after participation in music.

Data Analysis:

To empirically examine the effect of musical participation on self-efficacy, the data were analyzed using the SPSS statistical software according to the following procedures:

1) Reliability Analysis:

To verify the internal consistency of the self-efficacy measurement tool, Cronbach's α was calculated separately for the pretest and posttest. Reliability was analyzed in two formats: including all 12 items (10 standard GSES items + 2 custom

items) and excluding the 2 custom items. This dual approach allowed us to assess whether the instrument as a whole demonstrated sufficient consistency and stability.

2) Normality Test:

In preparation for paired-sample t-tests, the difference scores between pretest and posttest were tested for normality using the Kolmogorov-Smirnov test. Paired-sample t-tests were only applied if the assumption of normal distribution was met.

3) Paired-Sample t-Test:

To examine whether the difference in mean self-efficacy scores before and after musical participation was statistically significant, a paired-sample t-test was conducted. The test was applied to both the total scores and the individual item scores to identify specific areas of self-efficacy that may have improved.

4) Item-Wise Score Change Analysis:

For each of the 12 GSES items, the mean difference between pre- and posttest scores was calculated. These results were visualized using graphs and tables to highlight which psychological domains showed the greatest changes in self-efficacy. This exploratory analysis provided insight into the specific psychological dimensions affected by musical participation.

5) Correlation Analysis Between Items:

To explore the structural relationships among subcomponents of self-efficacy, Pearson correlation coefficients were calculated between individual items. This helped identify patterns of interaction among sub-factors and revealed whether certain response tendencies were shared across items.

6) Correlation Between Initial Scores and Change:

To determine whether participants with lower initial self-efficacy benefited more from the musical experience, a Pearson correlation was computed between the pretest scores and the difference scores (posttest – pretest). This allowed us to examine whether those with lower starting levels of self-efficacy experienced greater gains.

Results and Discussion

Normality Test Results:

To assess whether the differences in self-efficacy scores before and after musical participation followed a normal distribution, a Kolmogorov-Smirnov test was conducted. The results are presented in Table 3.

Table 3: K-S Statistic and p-Value. The difference scores met normality ($K-S = 0.136$, $p = .592$), validating the use of parametric tests for mean change.

K-S Statistic	p-Value
0.136	0.592

The Kolmogorov-Smirnov (K-S) test is a non-parametric method used to evaluate whether a given dataset follows a specific distribution—in this case, the normal distribution. This test is commonly employed to validate the assumptions required for parametric statistical analyses.

In this study, the test was applied to the difference scores between the posttest and pretest self-efficacy measurements. The result showed a K-S statistic of 0.136 and a p-value of 0.592, which exceeds the conventional significance threshold of 0.05.

Therefore, it can be concluded that the difference scores are normally distributed. Based on this result, a paired-sample t-test was subsequently conducted to examine whether the mean change in self-efficacy before and after musical participation was statistically significant.

Paired-Sample t-Test Results:

To determine whether the change in self-efficacy following musical participation was statistically significant, a paired-sample t-test was conducted. The results are shown in Table 4.

Table 4: Paired-Sample t-Test Results. Self-efficacy increased by 0.55 points on average (pre: 3.20; post: 3.75), a highly significant gain ($t(29) = -4.58$, $p < .001$).

Variable	Pretest M (SD)	Posttest M (SD)	Difference M (SD)	t	p
Musical Participation	3.20 (0.66)	3.75 (0.75)	0.55 (0.73)	-4.58	.00008

To examine the effect of musical participation on adolescents' self-efficacy, a paired-sample t-test was conducted on the mean self-efficacy scores before and after the activity. The analysis revealed that the mean pretest score was 3.20 (SD = 0.66), and the mean posttest score was 3.75 (SD = 0.75). The average increase in scores was 0.55 (SD = 0.73).

The paired t-test yielded a statistically significant result, $t(29) = -4.58$, $p < .001$, indicating that the increase in self-efficacy following musical participation was highly significant. This suggests that the improvement was unlikely to have occurred by chance.

Additionally, despite the standard deviations being similar between pretest and posttest scores (0.66 and 0.75, respectively), the significant increase in the mean score implies that the musical activity may have contributed to a genuine psychological enhancement rather than a temporary or situational response.

Item-by-Item Score Change Analysis:

To examine the change in self-efficacy across individual items more specifically, the difference in mean scores (posttest – pretest) for all 12 items is visualized in Figure 3. This analysis was used to explore and interpret how musical participation influenced the subcomponents of self-efficacy.



Figure 3: Changes in Item-Level Self-Efficacy Scores Before and After Musical Activity (Posttest – Pretest Mean Scores). Largest gains appeared in stage confidence (Item 11, $\Delta M = 0.90$), stress regulation (Item 9, $\Delta M = 0.80$), and multitasking (Item 8, $\Delta M = 0.77$), while calmness under difficulty (Item 3) changed least.

According to the figure, Item 11 (“I feel confident singing or acting on stage”) showed the largest increase in mean score ($\Delta M = 0.90$), which can be interpreted as being directly related to the nature of musical activities. This result suggests that opportunities for self-expression, stage experience, and perceived success during performance positively contributed to enhanced self-efficacy.

In addition, Item 8 (“I believe I can manage several different tasks at the same time”) and Item 9 (“I believe I can manage and regulate stress well”) also demonstrated notable increases in scores ($\Delta M = 0.77$ and $\Delta M = 0.80$, respectively). These results suggest that the multifaceted nature of musical experiences, including script memorization, stage rehearsal, time management, and collaboration, contributed to improved self-perception in areas such as executive function and emotional regulation.

On the other hand, Item 3 (“I can remain calm even in difficult situations”) showed a relatively smaller change ($\Delta M = 0.27$), suggesting that certain emotional stability traits may be less susceptible to short-term interventions.

Overall, the item-level score patterns indicate that musical participation had especially positive effects on self-expression, emotional expression, stress regulation, and the ability to handle complex tasks.

Item-to-Item Correlation Analysis:

To examine the interrelationships among the sub-items of the self-efficacy scale, a Pearson correlation analysis was conducted. The analysis was based on a total of 60 observations, integrating both pre- and post-survey responses. The goal was to explore the similarity in response patterns across items and to identify structural associations among the subcomponents of self-efficacy. The results are shown in Figure 4.

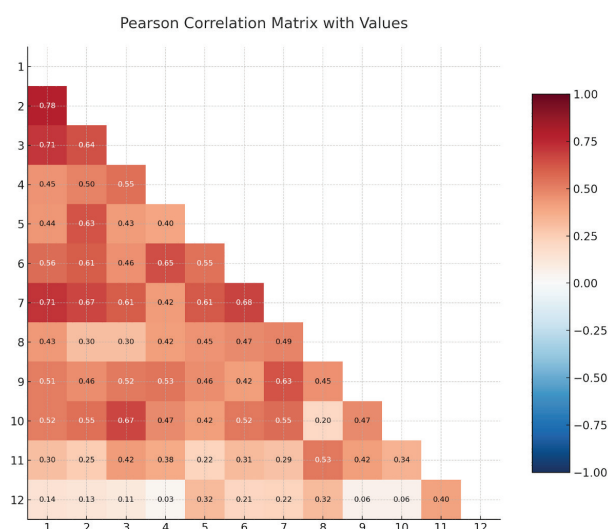


Figure 4: Correlation matrix between items. Core cognitive-behavioral efficacy items showed moderate-to-strong associations (e.g., Item 1–2 $r = .78$), whereas stage confidence (Item 11) and emotional expression (Item 12) were more weakly linked, indicating context-specific components.

The results indicated predominantly positive correlations between most item pairs, with the following item pairs showing particularly strong correlations:

- Item 1 (Ability to solve unexpected difficulties) – Item 2 (Solving important problems): $r = 0.78$
- Item 1 – Item 7 (Overcoming obstacles): $r = 0.71$
- Item 3 (Staying calm in difficult situations) – Item 10 (Sense of control over life): $r = 0.67$
- Item 2 – Item 5 (Persistence when facing challenges): $r = 0.63$

These findings suggest that problem-solving ability, self-regulation, goal persistence, and a sense of control are closely interrelated within the self-efficacy construct. In particular, the cognitive and behavioral efficacy domain (Items 1–7 and 10) showed generally moderate-to-strong correlations ($r \geq 0.5$), supporting the construct validity of the scale.

In contrast, Item 11 (Confidence on stage) and Item 12 (Ease of emotional expression) showed weaker correlations with most other items. For example, Item 12 was only weakly correlated with Item 1 ($r = 0.14$) and Item 2 ($r = 0.13$). This suggests that these items reflect distinct, experience-based psychological factors that differ from general cognitive components of self-efficacy. In the context of musical participation, they may function as independent psychological constructs.

Correlation Between Pre-Test Scores and Score Changes:

To analyze whether the impact of musical participation on self-efficacy improvement varies depending on initial levels of self-efficacy, a Pearson correlation analysis was conducted between the pre-test mean scores and the score changes (post-test minus pre-test). The analysis yielded a correlation coefficient of $r = -0.35$ with a p -value of .057.

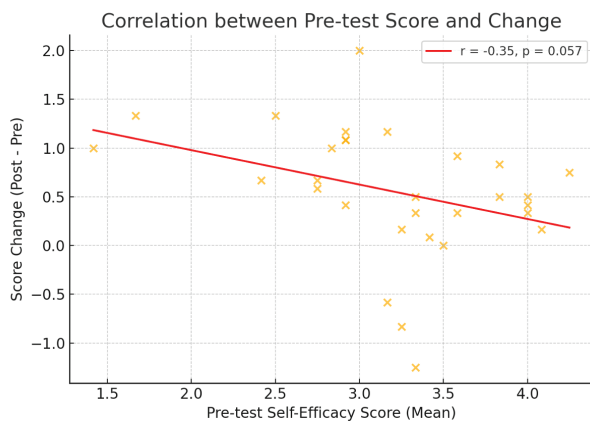


Figure 5: Scatterplot of pre-test self-efficacy scores and score changes. Score change trended negatively with baseline ($r = -.35$, $p = .057$), suggesting students starting lower tended to improve more after rehearsal.

Figure 5 presents a scatterplot showing each participant's pre-test self-efficacy score (x-axis) and the corresponding change in score (y-axis). The regression line in the scatterplot has a negative slope, indicating a general trend: participants with lower initial self-efficacy scores tended to show greater improvements. For example, students whose initial scores were in the 2-point range often recorded increases of over 1 point. In contrast, those with already high pre-test scores (e.g., above 4 points) exhibited minimal or no change.

Although the p -value of .057 is slightly above the conventional significance level of 0.05, making it difficult to assert a

statistically significant correlation, the negative trend remains observable. This trend may serve as preliminary evidence that musical participation could be a more effective intervention for students with lower initial self-efficacy. Further studies with larger sample sizes are needed to validate this finding.

Conclusion

Summary and Interpretation of Findings:

This study quantitatively analyzed the impact of musical participation on adolescents' self-efficacy and aimed to examine the psychological structure and characteristics of that effect. A one-shot pretest–posttest design was applied to a sample of 30 high school students, and various statistical analyses yielded the following results.

First, the average self-efficacy score significantly increased after the musical activity ($t = -4.58$, $p < .001$), suggesting that musical experiences can positively influence adolescents' self-perception and emotional self-efficacy. In particular, item-level analyses revealed notable increases in stage confidence (Item 11), stress regulation (Item 9), and multitasking ability (Item 8). These findings suggest that musical participation can enhance self-efficacy through psychological self-regulation, emotional expression, and the execution of complex tasks.

Correlation analysis between items revealed significant positive relationships among subcomponents of self-efficacy, especially strong correlations between problem-solving abilities (Items 1, 2, 7) and persistence toward goals. In contrast, items related to stage confidence (Item 11) and emotional expression (Item 12) showed relatively lower correlations with other items, suggesting that these are specific psychological components more closely tied to the musical context.

Additionally, although not statistically significant ($r = -0.35$, $p = .057$), the negative correlation between pretest scores and score change suggests that students with lower initial self-efficacy experienced greater improvements. This trend suggests that musical participation may provide particularly powerful psychological reinforcement for adolescents with lower initial levels of self-efficacy.

Comparison with Previous Research:

These findings align with both Bandura's theory of self-efficacy and previous research on the psychological benefits of arts-based activities.

Bandura identified four sources of self-efficacy—mastery experience, vicarious experience, verbal persuasion, and physiological/emotional states—all of which were present throughout the participants' musical engagement.¹ Specifically, the rehearsal and performance processes provided mastery experiences, while the emotional tension and release surrounding performances contributed to affective gains, collectively enhancing self-efficacy.

The results are also consistent with studies such as Seong & Kim on orchestra participation, Jo & Koo on arts-based activities improving self-esteem and creativity, and Hughes & Wilson on the contributions of youth theaters to social de-

velopment.⁹⁻¹¹ These works collectively suggest that artistic activities promote adolescent psychological growth and identity formation. This study adds further empirical support by focusing on musical theater as a comprehensive artistic intervention.

Furthermore, the item-level correlation structure and sensitivity patterns correspond to Zimmerman's characterization of self-efficacy as context-dependent and multidimensional.⁴ The presence of items that responded more sensitively to the musical activity supports the theoretical view that situational and task-specific factors influence self-efficacy.

The observed tendency for students with lower baseline self-efficacy to show greater gains echoes Bandura's assertion that self-regulation is reinforced through repeated success experiences, as well as Kim's findings on the relationship between self-efficacy and job competence, and Zareai's study on how self-efficacy influences interpretation quality.^{2,3,5} These parallels suggest that arts-based interventions may be especially effective for students with initially lower confidence, offering important educational implications.

Limitations and Significance:

However, this study has several limitations:

First, the sample consisted of 30 students from a single high school who voluntarily participated, limiting the generalizability of the findings. Variations based on region, socioeconomic background, or educational environment were not considered, indicating a need for future studies with larger, more diverse samples that include adolescents from varied socioeconomic backgrounds, geographic regions, and school types (e.g., public vs. private, urban vs. rural), in order to be context-dependent.

Second, the use of a one-shot pretest–posttest design without a control group limits the ability to attribute the observed improvements solely to the musical activity. External factors such as personal motivation or other life events may have influenced the outcomes. Future studies should incorporate a control group engaging in a non-musical structured activity of similar duration (e.g., a focused reading or art-drawing session) to isolate whether the observed self-efficacy gains are specific to musical participation or a broader effect of any engaging extracurricular experience.

Third, the study did not assess the long-term effects or sustainability of the observed improvement. Therefore, it remains unclear how lasting the impact of a one-time musical experience is. Future research should incorporate structured follow-up assessments administered at one day, one week, and one month post-intervention to evaluate whether the gains in stage confidence, stress regulation, and multitasking ability are sustained over time or represent only a momentary psychological response to the activity.

Despite these limitations, this study has meaningful implications, presenting quantitative evidence of the effectiveness of arts-based interventions in improving adolescent self-efficacy. It affirms that musical theater—a comprehensive and integrated art form—can make a positive contribution to identity formation and psychological growth during adolescence. These findings may serve as foundational data for designing

future school-based arts education programs or psychological intervention models. Provide a summary of the results of the research concisely.

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Artificial Confidence: A Secondary Data Analysis of AI-Labeled Advice and Cognitive Effects Across Age Groups

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ABSTRACT: Artificial intelligence (AI) is being rapidly adopted in decision-making in critical sectors such as health, education, and public services. However, there is a paucity of research assessing the differential impact of AI- or human-labeled advice on human cognition and resulting behavior. We attempted to understand AI's impact on human decision-making through a secondary data analysis of the Human-AI Interactions Dataset, consisting of approximately 34,000 decision-making records. We used two outcome measures: confidence drift (the alteration in self-reported confidence from pre- to post-advice) and decision change (the user's revision of their answer). Results revealed that AI-labeled advice significantly increased confidence drift relative to human advice ($p < .00001$). However, the increase in confidence did not correspond to a higher likelihood of decision change. Age also emerged as a significant negative predictor of confidence drift and decision change. However, the interaction between age and advice source was not statistically significant. The study results suggest that while AI advice may increase users' perceived decision certainty, it does not change actual behavior. Hence, to improve human-AI collaboration, future AI systems should focus on trust calibration, age sensitivity, and the promotion of critical thinking without inducing overconfidence.

KEYWORDS: Behavioral and Social Sciences, Cognitive Psychology, Human-AI Interaction, Decision Making, Trust Calibration.

■ Introduction

The adoption of artificial intelligence (AI) in decision-making processes has accelerated across our society. AI-based decision-making is deployed in healthcare, education, and governance. Arguably, AI systems deliver efficiency and convenience. But they also raise concerns about the influence of AI-generated advice on human responses. One of the important factors determining this response is trust. AI systems develop through a combination of dispositional tendencies, situational context, and learned experience over time, reflecting both cognitive evaluations (e.g., perceived reliability and competence) and affective responses.¹ Trusting AI dictates how individuals rely on AI recommendations, often irrespective of its accuracy.^{2,3} Trust can evolve and may become miscalibrated, leading to either over-reliance on incorrect AI or rejection of correct advice.⁴

Many recent studies have examined cognitive challenges for AI users in decision-making. Srinivasan and Thomason⁵ proposed adaptive systems to recalibrate trust in AI to improve human-AI collaboration. Alon-Barkat and Busuioc⁶ emphasized the importance of users' pre-existing beliefs that bias them to adhere to AI advice. One of the classic works in the field by Yaniv and Kleinberger⁷ demonstrates that individuals incorporate external advice only partially, often ignoring even accurate recommendations in favor of their own initial judgment. This phenomenon is known as egocentric discounting. Persistent cognitive tension between personal judgment and external input from AI should be examined carefully to make human-AI collaboration more trustworthy. However, current literature largely focuses on behavioral outcomes rather than internal psychological effects.⁸ This study addresses that gap

by examining whether AI-labeled advice increases users' confidence more than identical human-labeled advice and whether this effect varies across age groups. Using over 34,000 decision-making records from the Human-AI Interactions Dataset, this paper contributes to understanding how AI affects user cognition by focusing on confidence drift as a key outcome measure.⁹ This study specifically compares AI-labelled and human-labelled advice and does not examine conditions of complete advice rejection, which remain an important area for future research.

Several theoretical models have been developed to explain the variability of trust in AI and its impact on user engagement. Hoff and Bashir¹⁰ proposed a three-layered model of trust variability. It is divided into dispositional (enduring tendency), situational (context-dependent), and learned (based on experience). They emphasize that facilitating appropriate trust is critical to improving safety and productivity in human-automation teams. Arguably, both over-trust (misuse) and under-trust (disuse) can lead to adverse events. Jacovi *et al.*³ introduced "contractual trust," where users evaluate AI systems not only by outcome accuracy but by perceived consistency with expected behavior. They also mention the concept of "warranted trust," which is ethically desirable as compared to "unwarranted trust," which can lead to misuse or disuse.

A significant increase in the use of automation has not always led to comparable improvements in efficiency. Hence, researchers have analyzed the importance of trust calibration in human-AI systems. Dzindolet *et al.*² found that initial positivity bias toward unfamiliar AI systems can erode rapidly. Particularly after encountering errors, unless the AI system provides comprehensible explanations, trust can rapidly di-

minish. While Dzindolet *et al.*² demonstrate that errors reduce trust in automated systems, the influence of error severity is not explicitly examined and may also play an important role, with more serious or high-stakes errors likely to result in greater reductions in trust than minor inaccuracies. On the contrary, knowing why the AI might err was vital, as it made people trust and rely on it more. Parasuraman and Riley⁴ identified four modes of automation interaction: use, misuse, disuse, and abuse. They argued that inappropriate trust accounts for serious automation-related failures. Vodrahalli *et al.*⁹ conducted a large-scale study using the Human-AI Interactions Dataset. Their findings suggested that participants accepted AI-labeled advice 5–6% more often than identical human-labeled advice, with small task-specific variation. Their work focused mostly on advice acceptance rather than confidence modulation or age-based effects.

Beyond system performance, individual differences like demographic factors can also shape how users engage with automation. Czaja *et al.*¹¹ studied the adoption of technology in the adult population (ages 18–91). They found that older adults are significantly less likely to adopt new technologies. Reduced self-efficacy, lower fluid intelligence, and computer anxiety were the factors influencing older adults. Another study by Pak *et al.*¹² had similar findings, highlighting the significance of age. Younger students effectively calibrated their trust with automation reliability, while older adults and cadets exhibited higher overall trust regardless of reliability. These findings emphasise the need for age-aware AI system design.

Taken together, these findings show that trust formation is a complex process, influenced by both the system design and human cognition. Researchers have explored cognitive interventions to correct such overreliance. Bućinca *et al.*¹³ demonstrated that "cognitive forcing interventions" significantly reduced overreliance on AI. The participants tend to make significantly more correct decisions on incorrect AI predictions. They suggested that human cognitive motivation can moderate the effectiveness of explainable AI solutions. Srinivasan and Thomason⁵ found that both excessively low and high trust levels lead to inappropriate AI reliance. Doctors accepted 26% of AI misdiagnoses when trust was high versus only 8% when trust was lower. They also found that providing supporting and counter-explanations resulted in a 38% reduction in inappropriate reliance and a 20% improvement in decision accuracy. Existing literature establishes the importance of trust and advice-taking in human-AI interactions. The development of trust may also depend on an individual's prior knowledge or expertise in the domain, as users with limited understanding may be more likely to either over-rely on or under-trust AI advice due to uncertainty. But most of them didn't examine internal psychological effects sufficiently. This study stresses confidence drift (change in the participant's confidence) as a key outcome measure, giving a more nuanced perspective on AI's impact on user cognition in decision support systems. This study tests the following hypotheses: H1: AI-labelled advice will produce greater confidence drift than human-labelled advice. H2: AI-labelled advice will increase the probability of

decision change. H3: Age moderates the effect of advice source on both confidence drift and decision change.

■ Methods

We conducted a secondary data analysis of a publicly available Human-AI Interactions Dataset developed by Vodrahalli *et al.*⁹ This study examines how AI-labeled advice affects confidence drift and whether age moderates this cognitive response, both of which remain underexplored.

Dataset and Participants:

The dataset comprised 35,670 decision-making trials contributed by 1,141 adults. The participants were recruited through Amazon Mechanical Turk. They were asked to complete a series of cognitive (decision-making) tasks. Tasks were spread across four domains: art authentication, city prediction, census prediction, and sarcasm detection. The participants had one initial response, and then they viewed the advice that was randomly labeled as either "AI-generated" or "human-provided". Participants also provided a confidence rating for their initial response. They were then shown advice that was randomly labelled as either 'AI-generated' or 'human-provided', although the underlying advice content was identical across conditions. After viewing the advice, they submitted a final response. A total of 34,783 valid interactions were used for analysis after excluding incomplete responses. This design isolates the effect of advice labelling independent of content.

Variables:

The study used two key outcome measures, namely confidence drift and decision change. Confidence drift was defined as the numerical difference between post-advice and pre-advice confidence ratings (post – pre), where positive values indicate an increase in confidence after receiving advice. Decision change was defined as a binary variable (0 = no change, 1 = change), indicating whether participants altered their initial response after receiving advice. Two key predictors used were advice source (labeled as AI or human) and age. Age was measured both as a continuous and categorical variable grouped into six ranges (25 years or younger, 25–34, 35–44, 45–54, 55–64, and 65 years or older).

Statistical Analysis:

We used Datatab (www.datatab.net), an online platform for statistical analysis. Sample characteristics and outcome distributions were summarized with descriptive statistics. The difference in mean confidence drift between AI and human advice groups was measured by an independent samples t-test. A two-way ANOVA was used to examine the interaction effect between advice source and age group on confidence drift. Multiple linear regression was used to predict confidence drift as a function of age, advice type, and their interaction. Finally, a logistic regression model assessed the likelihood of decision change based on age and advice source.

Ethical Considerations:

Dataset by Vodrahalli *et al.*⁹ is licensed under the Creative Commons Attribution 4.0 License. No additional ethics approval was required for this secondary analysis since the dataset is anonymized and previously collected for research purposes.

Results

This study analyzed 34,783 interaction records from the Human-AI Interactions Dataset to examine the influence of AI versus human advice on user confidence and decision changes.

Confidence Drift by Advice Source:

An independent samples t-test was conducted to compare confidence drift between participants who received advice labeled as coming from an AI versus a human. The analysis showed that participants in the AI condition experienced significantly greater confidence drift ($M = 0.092$, $SD = 0.40$) than those in the human condition ($M = 0.074$, $SD = 0.37$), $t(34,700) = 4.46$, $p < .00001$ (Table 1). Although this result was statistically significant, the effect size was small (Cohen's $d = 0.048$). Given the large sample size, even very small effects can achieve statistical significance; therefore, this finding is unlikely to reflect a Type I error but instead indicates a statistically reliable yet practically small effect.

Table 1: Participants receiving AI-labeled advice experienced slightly higher confidence drift than those given human-labeled advice.

Advice Source	Mean Confidence Drift	Standard Deviation
AI	0.092	0.40
Human	0.074	0.37

Confidence Drift by Age Group and Advice Source:

A two-way ANOVA was conducted to examine the effects of advice source, age group, and their interaction on confidence drift. As shown in Table 2, there was a significant main effect of advice source, $F(1, 34,771) = 19.06$, $p < .00001$, and a significant main effect of age group, $F(5, 34,771) = 5.34$, $p < .0001$. The interaction between advice source and age group was marginal, $F(5, 34,771) = 2.06$, $p = .067$. These findings suggested a modest trend that different age groups may respond differently to AI versus human-labeled advice, although this effect did not reach statistical significance.

Table 2: Two-way ANOVA results show that both advice source and age group significantly influenced confidence drift, while their interaction was not statistically significant.

Source of Variation	F-value	p-value
Advice Source	19.06	< 0.00001
Age Group	5.34	< 0.0001
Advice Source x Age Group	2.06	0.067

Predicting Confidence Drift:

A multiple linear regression was conducted with age, advice source (coded 0 = human, 1 = AI), and their interaction entered as predictors of confidence drift. As shown in Table 3, age was a significant negative predictor, $\beta = -0.0011$, $p < .001$, indicating that older participants experienced smaller changes in confidence. Advice source and the interaction term

were not statistically significant. Overall, the analysis showed a very small proportion of variance ($R^2 = 0.001$), suggesting that while age was associated with confidence drift, AI labeling itself was not a strong independent predictor.

Table 3: Multiple regression results indicate that age negatively predicted confidence drift, whereas advice source did not have a significant independent effect.

Predictor	Coefficient (β)	p-value
Age	- 0.0011	< 0.001
Advice Source (AI)	+ 0.0020	0.880
Age x Advice Source	+ 0.0005	0.149

Predicting Decision Change:

A logistic regression was conducted to examine the likelihood of participants changing their answer after receiving advice. As shown in Table 4, age was a significant negative predictor, $\beta = -0.0029$, $p = .028$, indicating that older participants were less likely to change their decisions. Advice source and its interaction with age were not statistically significant, suggesting that the probability of changing answers did not differ meaningfully between AI and human-labeled advice.

Table 4: Logistic regression predicts a lower likelihood of decision change among older participants, regardless of whether the advice came from AI or human sources.

Predictor	Coefficient (β)	p-value
Intercept	+ 0.392	< 0.001
Age	- 0.0029	0.028
Advice Source (AI)	+ 0.056	0.366
Age x Advice Source	+ 0.0024	0.206

Multiple Comparisons and Statistical Limitations:

This analysis involved multiple statistical tests across two dependent variables and several interaction terms. No formal correction (e.g., Bonferroni) was applied due to the exploratory nature of the study. This approach is not without limitations, but it is consistent with methodological arguments by Rothman¹⁴ and Perneger¹⁵ that multiple-comparison adjustments can inflate Type II error rates and are inappropriate when tests address conceptually distinct hypotheses. Additionally, the primary finding in our analysis was highly significant ($p < .00001$), well below any reasonable correction threshold. Future studies should apply appropriate multiple-comparison corrections to ensure robust conclusions.

Discussion

We explored broader implications of AI-assisted decision-making through this study. Our findings support some of the earlier research and also highlight some interesting new dimensions of human-AI collaboration. Our study results confirmed that AI-labeled advice has a measurable cognitive impact on users, even though both groups received identical information. Those with AI-labeled advice expressed significantly greater confidence drift than those given human-labeled advice. Among the hypotheses tested, H1 was supported, as AI-labelled advice significantly increased confidence drift. However, H2 and H3 were not supported, as AI-labelled advice did not significantly influence decision change, and no significant interaction between age and advice source was observed. This finding is consistent with research by Zhang *et al.*,¹⁶ who found that AI framing and confidence signals

can disproportionately increase user trust without a change in performance. Our results extend those findings by demonstrating a clear dissociation between confidence changes and behavioral changes. AI-labeled advice increased participants' confidence, but there was no reciprocal change in decisions. One possible explanation for this dissociation is that AI-labeled advice may increase perceived authority or credibility, leading to greater subjective confidence without necessarily altering underlying decision processes. This may reflect cognitive anchoring, where individuals retain their initial judgments while adjusting confidence levels, or differences in how machine-generated advice is interpreted compared with human advice. Such dissociation means users feel more certain internally without necessarily making different decisions. One may characterize the irrational boost in user confidence via AI labeling as "artificial confidence". This psychological phenomenon suggests that AI advice has a limited effect on our behavior. This can have serious real-world implications with rising AI adoption. This finding is in contrast to some of the established automation bias literature. Most studies suggest that AI effects typically manifest as both cognitive and behavioral changes.^{2,4} A systematic review of automation bias by Goddard *et al.*¹⁷ also suggested that inappropriate reliance typically affects both decision-making processes and outcomes. Increased confidence without behavioral flexibility could also foster reduced openness to feedback and limit post-decision reflection in real-world applications.

We have considered several psychological mechanisms that may explain this dissociation between cognitive and decisional processes. From a cognitive perspective, AI labels may activate heuristics that enhance subjective certainty via algorithmic appreciation. This results in trusting algorithmic judgments more than human ones.¹⁸ This increased confidence is just through perceived legitimacy rather than improved understanding, which obviously doesn't result in a change in decision. AI labeling may also trigger responses through emotional pathways. Most importantly, technology optimism bias and automation complacency can create positive emotional responses to AI-generated content.¹⁹ Such an emotional boost may manifest as increased confidence without necessarily improving analytical thinking or behavioral flexibility. Both cognitive and emotional pathways may inflate confidence while leaving decision processes unchanged. This dissociation can be hypothesized through the dual-process theories of cognition.²⁰ AI labeling may primarily influence System 1 responses (fast, intuitive confidence feelings) while leaving System 2 processes (slow, deliberative decision revision) relatively unaffected. This explains why participants feel different but act similarly.

The small effect size (Cohen's $d = 0.048$) of our study needs further explanation, as even a small effect can amplify when applied to large populations or accumulate over time.²¹ In real-world scenarios, millions of users interact with AI systems daily. Small systematic confidence biases can aggregate to significant population-level effects. For instance, AI medical triage systems that modestly increase user confidence could influence help-seeking behavior across thousands of patients. Moreover, users rarely interact with AI systems only once.

Hence, small confidence increases may compound over repeated interactions, potentially leading to substantial shifts in trust and reliance patterns, as suggested by previous research.⁴ The confidence-behavior gap also raises particular concerns for AI deployment in high-stakes fields. In particular, users who feel more confident without improved decision quality may be less likely to seek additional information, consult experts, or engage in critical evaluation, resulting in potentially dangerous consequences. These findings extend beyond AI's influence on decisional accuracy alone. They demand stronger AI transparency and optimized trust calibration alongside greater AI adoption.

The analysis also revealed meaningful age-related patterns similar to prior observations by Czaja *et al.*¹¹ and Pak *et al.*¹² Age consistently predicted lower responsiveness to advice in our study. Older adults showed smaller confidence drift and lower rates of decision change. However, the interaction between age and advice source did not reach statistical significance. This suggests that the effect of AI remains relatively similar across the age groups. Younger adults may be more cognitively malleable overall, but AI-labeled advice appears to influence confidence consistently, regardless of age. Our findings urge designers to recognize AI's impact on cognition, which seems to be more pronounced than its effect on behavior. This might also be particularly important to younger users who are likely to adopt AI more readily and may therefore be more vulnerable. On the other hand, for older adults, building initial trust may be more important than simply improving algorithmic accuracy. As Rahwan *et al.*²² emphasize, understanding how AI systems influence human cognition and decision-making becomes crucial as machine behavior increasingly shapes our societies. Recent research explores these concerns in sectors like mental health and education. Zhai *et al.*²³ established that AI tools designed for mental health triage in higher education can shape student self-assessment even without any behavioral intervention. Similarly, educators appreciate AI for a personalized learning experience but express concern about students' overdependence on AI-generated feedback.²⁴ Hence, these findings may point to the potentially serious cognitive risks posed by AI-induced confidence drift in real-world learning and support environments. Age-related differences in learning may also influence this effect, as younger individuals tend to demonstrate greater cognitive flexibility, whereas older individuals may rely more on established knowledge and heuristics. This may affect their ability to critically evaluate and adapt to AI-generated advice.

An overall analysis of our results is consistent with research in the field of trust in Human-AI collaboration. For example, research by Glikson and Woolley²⁵ describes emotional and cognitive trust, which highlights subconscious emotional over-reliance on AI systems. Similar research by Alon-Barkat and Busuioc⁶ also cautions against users' selective adherence to advice based on their pre-existing biases. If we inflate confidence artificially without meaningful flexibility in behavior, users may risk losing the ability to change behavior in response to their conviction. To mitigate these risks, Srinivasan and Thomason⁵ proposed adaptive AI systems to monitor user trust and adjust

their responses through more explanation or gentle cognitive challenges. In conclusion, future AI systems should encourage confidence, critical reflection, and behavioral change across all ages and contexts.

Limitations and Future Research Directions:

As this study is based on secondary data analysis, it is limited by the structure and variables of the original dataset. The researcher had no control over the experimental design, variable selection, or measurement processes, which may restrict the scope of interpretation. Despite relying on a large dataset, several important variables are ignored. Most importantly, the dataset fails to capture measures of education, AI familiarity, digital literacy, technology anxiety, and baseline trust. These variables are likely to influence cognitive effects. The study population included a primarily English-speaking Western sample, but cultural attitudes toward technology and authority can vary significantly across societies. Hence, results are not generalizable across cultures. The analysis also examined single-session interactions. Therefore, it is not representative of real-world AI use, which involves repeated exposure over time. Future longitudinal study designs should examine the evolution of confidence effects and determine if minor confidence enhancements compound into significant trust miscalibration over a period. Future studies should incorporate multiple comparison corrections and experimental designs that can manipulate AI labeling while controlling for actual advice quality. This can strengthen causal inferences about labeling effects and improve generalizability.

Conclusion

Our study shows that AI-labeled advice increases user confidence modestly compared to human-labeled advice, despite both providing the same information. But the cognitive process of a rise in confidence does not lead to greater behavioral flexibility or changes in decision-making. The relative effect of AI versus human advice remained consistent across all age groups, even though age came out as an important negative predictor of shift in confidence and change in decisions. These findings raise concerns about the growing use of AI-based advice in areas where decision accuracy is critical, such as healthcare, education, and public policy. Finally, the findings of this study highlight the need for AI systems designed to build trust while still encouraging critical reflection and independent judgment.

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Design and Experimental Studies for a PDMS Microfluidic Sensor Detecting Low Blood Flow Rate

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ABSTRACT: This study investigates whether Lorentz-force-based sensing—well established at the macro scale—remains valid at the micro scale within a PDMS microfluidic environment. We hypothesize that when an ionic solution flows through a PDMS microchannel in a magnetic field, the induced voltage will follow the same proportional relationship to flow velocity predicted by the Lorentz force equation in macro systems. To test this, we fabricated a PDMS microfluidic sensor with gold capacitor electrodes and introduced a potassium chloride (KCl) solution at controlled flow rates. Measured voltages were compared with theoretical predictions from Lorentz-force equations derived for macroscopic conductive fluids. Experimental results confirmed a strong linear correlation between measured and predicted voltages, with an average flow-rate error of 9.0% and the detectable flow rate minimum (0.028 mL/s). These results indicate that Lorentz-force behavior scales consistently down to microfluidic dimensions, validating the physical model's applicability at small scales. The findings demonstrate that Lorentz-force-based sensing can accurately quantify micro blood flow, providing a foundation for developing wearable cardiovascular monitoring technologies. This microfluidic sensor could provide valuable insight for future work.

KEYWORDS: PDMS, Microfluid Sensor, Lorentz Force, Flow Rate, Microfluidic Channel.

■ Introduction

Cardiovascular disease is the leading cause of death worldwide, responsible for 17.9 million deaths annually. Studies show that nearly 75% of these deaths could be prevented with early and continuous monitoring. Blood flow rate is an important physiological parameter to indicate the health of the cardiovascular system, especially related to blockages, and could provide insight into a variety of pathological conditions. Measuring blood flows allows for real-time monitoring of cardiovascular health, helping to indicate conditions such as thrombosis, arterial stenosis (narrowing), vessel occlusion, and arterial hardening.¹ Traditional methods for detecting blood flow rate include Doppler and pulse Doppler flowmetry (for large vessels) and laser Doppler flowmetry (for small vessels).² However, these methods require a clinical setting, are expensive and complex, and cannot offer real-time monitoring in patients' daily lives. This research focuses on microfluidic technologies for real-time, cost-effective, minimum-invasive blood flow rate monitoring using PDMS-based chips integrated with capacitors.

The development of PDMS microfluidic sensors has been seen as a significant breakthrough in the advancement of lab-on-a-chip technology, providing the ability to manipulate and analyze fluids at the microscale in real time with high precision and low cost.³ PDMS is an elastic polymer that is both flexible and biocompatible, and such high mechanical and chemical stability makes it popular in the microfabrication and bioengineering fields.⁴ In addition, PDMS is also a cost-effective material, with an average cost of \$3 per 1000 grams. PDMS's flexibility also allows the fabrication of deformable structures that are essential for creating biomedical devices.⁵ Given all

these qualities, we conclude that PDMS is highly suitable for the development of microfluidic sensors that are sensitive and scalable for human blood flow rate detection.

This study aimed to use PDMS material to develop a microfluidic sensor that could detect micro blood flow rate in contrast to traditional methods to measure blood flow rate, such as using large medical devices like Laser Doppler Flowmetry (LDF) and Pulse Doppler flowmetry (PDF). Utilizing a PDMS-based microfluidic sensor could provide a more cost-effective, convenient, and real-time monitoring for patients, making life easier for people suffering from cardiovascular diseases, reducing the number of follow-up visits after they have been diagnosed, and helping doctors to make corresponding adjustments to medications more conveniently. Existing microfluidic sensors for blood flow detection, each with limitations such as limited spatial resolution, high sensitivity to environmental changes, or expensive equipment, are mostly based on optical methods, capacitive sensing, or thermal anemometers.⁶ To overcome these barriers, recent works have suggested using resistive sensing techniques where changes in electrical fields are measured due to mechanical deformation or flow-induced changes. It uses flexible microchannels filled with conductive composites to provide a high response to changes in flow rate.⁷ Previous research has also shown that microfluidic sensors could be used to detect human vital signs such as blood pressure, as they have a biocompatible stability and are easy to wear.⁸ Therefore, we hypothesize that a flexible PDMS microfluidic chip embedded with conductive composite sensors can accurately and continuously measure blood flow rate, offering a practical tool for cardiovascular monitoring.

Inspired by previous research, we conclude that the PDMS-based flexible microfluidic sensor with integrated conductive composites could offer the most precise detection of flow rate changes; and due to the characteristics of microfluidic sensors and PDMS—such as biocompatibility, cost-effectiveness, ease of analysis, and precision—this system presents a highly promising platform for real-time, noninvasive cardiovascular monitoring and early disease detection. Based on this conclusion, we develop a flexible, PDMS-based microfluidic sensor combined with a conductive gold-circular capacitor following the Lorentz force law, as shown in Figure 1, which quantifies blood flow rate using electrical voltage measurements.

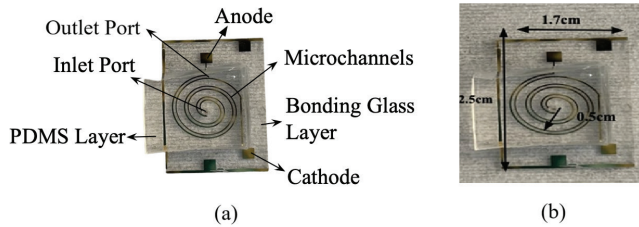


Figure 1: A flexible, PDMS-based microfluidic sensor combined with a conductive gold circular capacitor for micro blood flow detection. (a) The appearance and structure of the PDMS microfluid sensor. (b) The dimensions of the sensor.

■ Methods

The fabricated sensor is placed above a permanent magnet with a constant and uniform magnetic field. The blood analog test solution, potassium chloride (KCl), is dissolved in deionized water at a density of $\sim 1.060 \text{ g/cm}^3$ (blood density) to ensure adequate conductivity. The solution is pumped into the microfluidic sensor with a syringe pump at a constant flow rate. Figure 2 shows the electric force and magnetic force on the flowing ions of the solution in the microfluidic channel. Both the electric field and magnetic field can be defined from the Lorentz force equation:

$$\mathbf{F} = q\mathbf{E} + q\mathbf{v} \times \mathbf{B} \quad (1)$$

where $\mathbf{F}$ is the Lorentz force, q is the charge of the ions, $\mathbf{E}$ is the electric field, $\mathbf{v}$ is the flow velocity, and $\mathbf{B}$ is the magnetic field.

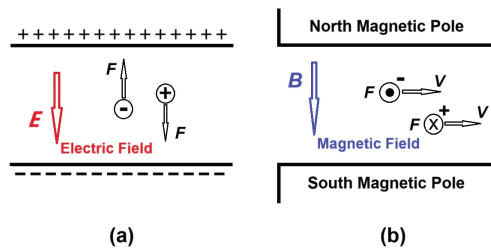


Figure 2: The electric force and magnetic force on flowing ions in the chip channel. (a) Electric force $F=qE$, a view from above of the flow channel. (b) Magnetic force $F=qv \times B$ perpendicular to both v and B , front view of flow channel.

Initially, the small flow channel consisting of two conductive walls and a non-conductive top and bottom is an uncharged coil capacitor. After the flow starts, the magnetic force pushes the positive and negative ions in opposite directions, and as the

positive and negative ions gather on the walls respectively, an electric field is formed.

$$E = -\frac{V}{w} \quad (2)$$

where V is the voltage across channel plates, and w is the channel width.

The volumetric flow rate Q is given by

$$Q = vA = vWh \quad (3)$$

where A is the cross-sectional area of the flow channel, and h is the height of the flow channel.

When the flow is in a steady state, the electric force is equal to the magnetic force in magnitude, but in opposite direction, so the Lorentz force in Eq. (1) is zero. Plugging Eq. (2) and Eq. (3) into Eq. (1), we obtain:

$$V = \frac{QB}{h} \quad (4)$$

In the experiment, the channel height h is $50 \text{ }\mu\text{m}$, and the magnetic field B is about 0.4 T . Using Eq. (4), the voltage across channel plates can be calculated after the volumetric flow rate is measured, or the volumetric flow rate can be predicted after the voltage across channel plates is measured.

Eq. (4) shows that the voltage is inversely proportional to the channel height. In the application of the chip channels as flow rate sensors, in order to obtain a stronger voltage signal, the channels should be made thinner. In this case, to avoid generating a large flow resistance, a wider channel can be used because the channel width does not affect the output voltage. On the other hand, if the chip channels are used as micro-electromagnetic pumps, higher height channels can produce higher flow rates at a given input voltage.

Wafer Preparation:

The PDMS microfluid sensor is fabricated with a standard photolithography and soft lithography process. As shown in Figure 3(a), a clean silicon wafer was prepared by rinsing thoroughly with deionized water, drying in a stream of clean air, and baking at 120°C for 10 minutes to remove any remaining moisture, allowing the SU-8 photoresist to stick better on the substrate.

Spin Coating SU-8 Photoresist.

The spin coating process is used to create the photoresist layer, which is going to be the mold. By applying SU-8 photoresist and by setting up the spin coater ramp at 500 rpm for 10 seconds, as shown in Figure 3(b), it generates the desired thickness of the photoresist film and lets the SU-8 photoresist spread over the silicon wafer homogeneously. The acceleration of the spin coater and the viscosity of the SU-8 photoresist will define the thickness of the layer we want.

Soft Bake:

After spreading the photoresist, the spin coated wafer is then subjected to a three-step soft bake on the hot plate at 65°C for 2 minutes, 95°C for 8 minutes, followed by again 65°C

°C for another two minutes, which helps to evaporate some of the solvents in the photoresist and also solidifies the wafer to prepare for UV exposure, as shown in Figure 3(c).

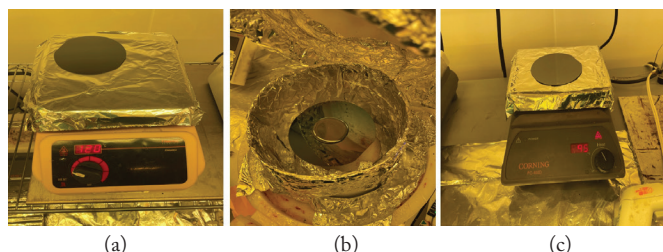


Figure 3: Wafer preparation and soft bake. (a) The wafer is rinsed with deionized water, dried with clean air, and baked at 120 °C for 10 min. (b) The wafer is coated with SU-8 photoresist using a spin coater. The spin coater's acceleration will control the thickness of the photoresist. (c) The wafer is soft-baked on a hot plate, 65 °C for 2 min, 95 °C for 8 min, and 65 °C for 2 min.

UV Exposure:

In the photolithograph process, a photomask with circular microfluidic channel patterns is aligned above the wafer, and exposed to SU-8 under UV light at 365 nm wavelength. As SU-8 is a negative photoresist, the areas exposed to electrons are cross-linked and thus become solid.

Post-Exposure Bake:

After UV exposure, the wafer is baked at 65 °C for 2 minutes, 95 °C for 8 minutes, and 65 °C for 2 minutes, with the same pattern as the soft bake. The post-exposure bake brings energy to continue the reaction activated by the UV exposure. The wafer was put into the SU-8 developer for 4–5 minutes to remove regions not exposed, and then rinsed with deionized water.

Development of the SU-8 Photoresist Mold and Bonding:

The wafer was put into the SU-8 developer for 4–5 minutes to remove non-linked photoresist, and then washed with isopropyl alcohol.

After the UV exposure of the silicon wafer, the PDMS is poured over the SU-8 mold and cured to produce the micro-channel layer. Concurrently, a gold layer was deposited and patterned into the capacitor-based sensing circuit on a glass substrate. The gold was chosen for the capacitor walls because it has good biocompatibility, conductivity, and corrosion resistance. The cured PDMS chip is peeled off the mold and bonded with the glass substrate under a microscope after plasma treatment to make sure that the microchannels are accurately aligned with the circular golden capacitor. Figure 4 shows the PDMS layers and the development of a golden circular capacitor.

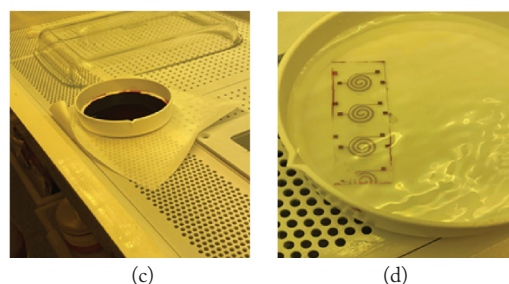
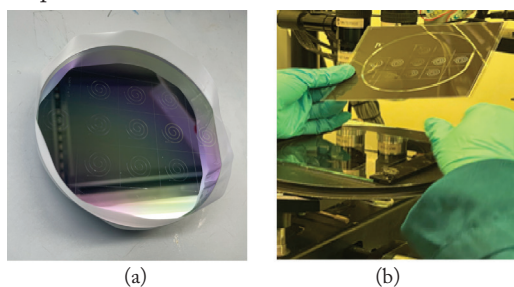


Figure 4: PDMS layers and the development of a golden circular capacitor. (a) Silicon wafer mold with PDMS on top. (b) The photomask with a circular microfluidic channel pattern is used in the photolithography process. (c) A gold circular capacitor before development. (d) The gold circular capacitor after development.

Experimental Methods:

The fabricated sensor is placed above a permanent magnet with a constant and uniform magnetic field of 0.4 T, measured with a Tesla meter. The blood analog test solution, potassium chloride (KCl), is dissolved in deionized water with a density of 1 g/cm³ to ensure adequate conductivity. The solution is pumped into the microfluidic channel by a syringe pump at a constant flow rate, as shown in Figure 5.

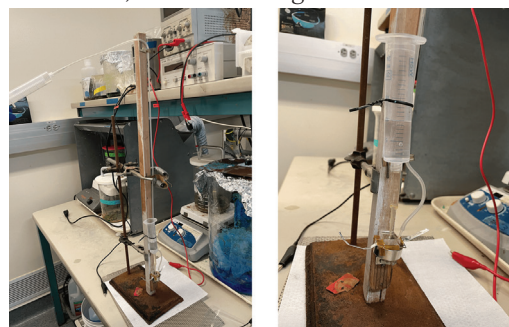


Figure 5: Experimental setup for the Lorentz-force measurements. The PDMS sensor is mounted above a permanent magnet while a syringe pump delivers ionic KCl solution at controlled flow rates. The induced voltage is recorded using a high-impedance differential electrometer.

Results and Discussion

To test the effectiveness of our PDMS-based microfluidic sensor in detecting micro blood flow rate, we conducted a series of experiments designed to test the sensor's accuracy in measuring fluid flow rates similar to those found in the human cardiovascular system. The rationale for these experiments was to determine whether the sensor could detect and quantify flow rates within a physiologically relevant range, while maintaining sufficient accuracy (<15% of error).

In the experiments, we use a syringe pump to introduce potassium chloride (KCl) solution dissolved at a density of ~1.060 g/cm³ (blood density) at known flow rates into the microfluidic channel of the sensor. The flow channel size is 0.4 mm wide and 0.05 mm high. The voltage between the anode and cathode was recorded during each trial. Using the Lorentz force law, we calculate the flow rate based on the detected voltage. We compared the measured flow rate from the sensor and the flow rate from the syringe pump and calculated the flow rate error for each trial (Table 1). The flow rates measured by

the PDMS microfluidic sensor have a good agreement with the pump flow rates, as shown in Figure 6.

Our results show that the given flow rate by the pump is from 0.028 mL/s to 0.286 mL/s, and the measured flow rate by the sensor is from 0.025 mL/s to 0.263 mL/s. This range covers the average blood flow rate within the human body, such as the average blood flow rate in arteries (median of 0.24 mL/s) and in veins (median of 0.05 mL/s), showing the biomedical applicability of the device.⁹ Meanwhile, the average flow rate error detected by the sensor is 9.0% (Table 1), which is within the 10% margin commonly accepted in biomedical engineering for wearable sensors for healthcare monitoring.¹⁰ This level of accuracy indicates that the sensor can potentially serve as a reliable wearable device for monitoring blood flow rate.

Table 1: Comparison of measured and reference flow rates. The table presents the pump flow rate, corresponding measured voltage, calculated flow rate from the Lorentz-force equations, and percentage error. The calculated voltage is derived from Eq. (4) using the given pump flow rate.

Test #	Given flow rate by pump [mL/s]	Measured voltage [mV]	Calculated voltage [mV]	Measured flow rate by sensor [mL/s]	Voltage error	Flow rate error
1	0.028	0.20	0.22	0.025	-10.0%	11.1%
2	0.067	0.50	0.53	0.063	-6.3%	6.7%
3	0.125	0.90	1.00	0.113	-10.0%	11.1%
4	0.214	1.60	1.71	0.200	-6.7%	7.1%
5	0.286	2.10	2.29	0.263	-8.1%	8.8%
				Average error	-8.2%	9.0%

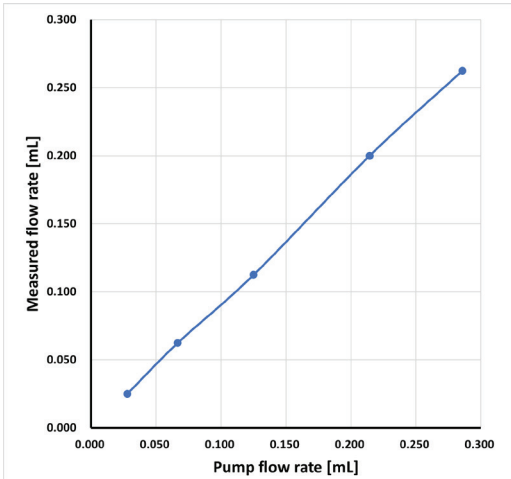


Figure 6: Correlation between measured voltage and flow rate. The plot shows linear dependence between voltage output and volumetric flow rate, demonstrating agreement with Lorentz-force theoretical predictions.

Additionally, the sensor’s compact dimensions (2.5 cm × 1.7 cm × 0.5 cm) meet the spatial constraints to be a wearable device for blood flow monitoring (Figure 1). Furthermore, the average cost per sensor is approximately \$22, indicating the cost-effectiveness of the design and the potential for mass production.

The results of these experiments confirm that the device successfully meets its design criteria: physiological relevance, measurement accuracy, compactness, and cost-effectiveness. Given the biocompatibility and flexibility of PDMS, along

with the sensor’s consistent accuracy in flow rate relations, our data support its potential for real-time monitoring of blood micro flow to aid in cardiovascular monitoring.

While the results confirm the sensor’s accuracy, several limitations must be acknowledged. First, testing was performed using a KCl solution in standard human blood density under controlled laboratory conditions, which does not fully guarantee the sensor’s performance in the more complex rheological and biochemical properties of blood, such as non-Newtonian viscosity and cellular components. These factors could affect flow dynamics and electrode response, causing a larger inaccuracy. Second, the experiments were conducted at room temperature; temperature variations in a clinical or wearable setting could affect PDMS elasticity and sensor performance. Third, while the average error was within the accepted biomedical threshold, the voltage signal measurement process requires clean cathode and anode surfaces; even tiny ions adhering to the electrodes could alter voltage measurements, causing the sensor’s performance to vary and result in a larger error in fluid flow rate reflection.

As the voltage signal detected is inversely proportional to the height of the channel, reducing the channel height could potentially improve signal strength, resulting in a stronger voltage signal and a more accurate fluid flow rate detected. Furthermore, as Eq. (4) shows, the width of the channel does not affect the voltage signal detected; expanding the width of the channel will reduce the resistance inside the sensor, increasing the accuracy of the overall detected flow rates.

These findings have a significant impact on the development of minimally invasive biomedical devices for real-time blood flow monitoring. The PDMS microfluid sensor’s ability to detect analog blood flow rates in the physiological range with less than 10% error suggests that the sensor could serve as a foundation for wearable health monitoring technologies. The small dimensions and low-cost characteristics of the design further demonstrate the sensor’s potential for large-scale production in both hospital and home-care environments, reducing the cost for cardiovascular disease monitoring and the redundant follow-up diagnosis to measure human vitals such as blood flow rates around a certain location in the human body. Beyond blood flow monitoring, similar sensor designs could be adapted for intravenous infusion rate tracking, early detection of vascular blockages, or industrial applications involving microfluidic devices such as micro-electromagnetic pumps. The Lorentz force-based voltage-flow relationship can also be applied to Magneto Hydro Dynamics (MHD) pumps,¹¹ chaotic stirrers,¹² and minute mixers.¹³

■ Conclusion

This study evaluates a PDMS microfluidic sensor that uses the Lorentz-force equation to detect micro flow rate according to the correlation between flow rates and voltages to estimate micro blood flow rates, with the ultimate goal of developing a wearable device for real-time blood flow monitoring. The testing procedure involves using a KCl solution at a controlled blood density at controlled flow rates using a syringe pump and comparing the measured flow rates to the syringe pump’s

given flow rates. The experiments demonstrated that the device detects volumetric flows between 0.025–0.263 mL/s with a mean flow error of 9.0% compared to given syringe-pump values. These results support the hypothesis that a compact, low-cost PDMS sensor can measure physiologically relevant microflow rates with biomedical-level accuracy (under 10% error) while keeping costs suitable for mass production.

Future work should focus on addressing the limitations, developing corresponding implementation methods, and implementing animal testing. This research only uses an analog human blood solution (KCl); therefore, future experiments need to employ human blood or blood analog fluids under physiological conditions to test the sensor's performance in a more physiologically relevant environment. Testing across a wider temperature range would help determine the thermal stability of the PDMS and circular gold circuit system. Currently, the measured voltage sensitivity is about 7.5 mV per mL/s. To improve voltage sensitivity without excessive flow resistance, microchannel dimension optimization—specifically reducing height and increasing width—should be investigated under a variety of experiments. Meanwhile, integration with wireless data transmission and the programming of corresponding applications need to be developed to achieve continuous remote monitoring of blood flow rates and relevant human vitals.

From a biomedical standpoint, animal testing using various blood densities and patient conditions needs to be implemented to further support and adjust PDMS microfluid sensors for blood flow monitoring. Statistical analysis of repeated measurements with larger sample sizes will provide a more detailed and accurate assessment of the sensor's reliability. Ultimately, translating this technology to clinical use would require relevant medical device regulations, extensive human trials that account for various cardiovascular conditions, patient comfort, and integration methods that relate to corresponding circumstances.

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Virtual Power Plant Incentive Strategy Based on the SIR Model and Evolutionary Game Theory

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ABSTRACT: As the global industries' need for new energy increases daily, more electrical engineers emphasize the benefits of the Virtual Power Plant (VPP), a digital coordination of distributed energy resources. By rationally adjusting electricity incentives and staggering electricity usage, the Virtual Power Plant reduces the power grid's burden and lowers the electricity price, maintaining power grid stability and benefiting collaborators. Such a positive effect is directly related to the size of VPP's collaborator base. This paper proposed a method to optimize the collaborator base while maximizing the profit of VPP through electricity incentives. The method originates from basic SIR models, integrates revolutionary game theory to reflect the variety of collaborators, and develops a bilevel model to illustrate the dynamic relationship among the incentive, the collaborator base, and VPP's profit. The paper also includes a case study of a local Chinese electricity department's VPP operation to demonstrate the superiority of this method in guiding VPP's incentives. The study shows that the proposed method can effectively increase the cooperation rate to 85% and the profit by 16.3% compared to before using the method. Such a result proves the method's reliability and effectiveness in maximizing VPP's profit.

KEYWORDS: Energy: Physical, Electricity, Virtual Power Plant, Incentives, SIR Model.

■ Introduction

Virtual Power Plant (VPP), a digital network of distributed energy sources, is becoming more pervasive around the world as the demand for clean energy is increasing. Normal VPPs have a centralized control structure, as shown in Figure 1, where individual energy resources and electricity consumers are directly connected to control coordination centers.¹ The coordination center serves to efficiently use energy, reduce burden on the energy grid, and ensure energy usage's stability and security by rationally allocating power supply for consumption and encouraging staggered electricity use.¹

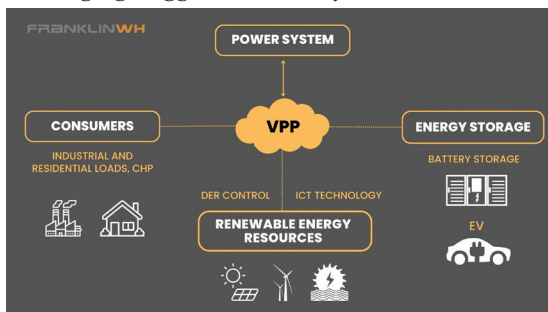


Figure 1: Virtual power plant diagram.² The centralized structure of VPP is shown by connecting the consumers and three different types of producers.

The effective allocation of energy resources enables VPPs to reduce power supply costs, enabling them to lower electricity prices below market levels to attract more consumers. Therefore, the proper functioning of VPP creates a positive spiral in which a larger consumer base gives VPP greater control over the energy grid, and greater control leads to more efficient energy prices. Commercial VPP profits in two ways: energy sales and government incentives.³ Governments provide incentives

to promote the adoption and integration of renewable energy.³ Again, a larger consumer base leads to better cooperation of the VPP; therefore, the expansion of collaboration is the essence of both the VPP and the consumer's profit.

One distinct way to foster collaboration is VPP's incentive to the collaborating enterprises.³ Much previous research has studied methods to analyze the economic relationship between VPP's pricing and market price.³ By using cooperative game theory, Yafei Wang, Weijun Gao, Fanyue Qian, and You Li analyze how to rationally allocate the profit from VPP operation to the demand and plant side, in forms such as incentives and tariffs, to ensure all participants are incentivized to join and maintain the long-term collaboration.⁴ Additionally, Zhengyu Shu and colleagues apply the Stackelberg game to create a bi-level model that demonstrates the market's reaction to VPP's pricing to satisfy demand and the effect of the reactions on VPP's final profit.⁵ However, current studies on consumers' reactions to VPP's incentive price often simplify consumers as homogenous individuals, overlooking the differences between them and their influence on each other's decision-making. Instead of assuming that consumers are completely and ideally rational when responding to incentive prices, research should analyze how collaboration can dynamically spread and collapse within a heterogeneous consumer base.⁶

Therefore, the researchers adopt SIR (Susceptible-Infected-Recovered) models as a foundation to simulate the dynamic changes, fueled by incentives, in collaborative relationships between consumer groups and the VPP. The SIR model is originally used in biomedicine to predict the spread of certain diseases. This article specifically uses the SIRS model, which contains the additional reinfection rate and is used

for diseases that only create temporary immunity.⁷ In the VPP scenario, the disease is the collaboration of consumers; the susceptible population is the potential collaborators; the infected population is the current collaborators; and the recovered population is the cooperation withdrawers. Therefore, integrating the SIR model can vividly illustrate the state of collaboration within the consumer base. However, the basic SIR model applies only to a homogeneous population, assuming constant infection, recovery, and reinfection rates.⁷ The researchers then develop a system for categorizing consumers and create dynamic, averaged rates by integrating evolutionary game theory, prospect theory, and the Capital Asset Pricing Model into the definition of the rates. Furthermore, researchers utilize a bilevel model to connect such dynamic representations of collaboration to the total profit of the VPP, creating a timely, effective guideline on the VPP's incentive pricing to maximize its profit; in this way, the proposed model solves the problem and uncertainty of VPP incentive pricing in the complicated, heterogeneous market and facilitates energy companies to more likely achieve their targeted profit.

■ Methods

Materials

The conducted study uses no physical materials. Instead, the main materials only include key academic theories and Google Colab for the model solving. The specific consumers' information in the case study is obtained from a virtual power plant operator in a city in western China.

Model Derivation:

- Reflection of consumers' heterogeneity and model inputs setup:

In order to precisely represent the heterogeneity of the consumer population, the researchers first categorize the consumers into large, medium, and small according to their electricity consumption.

In the case of the virtual power plant operator in a city in western China, an enterprise with 0-1 MWh electricity usage is considered small, an enterprise with 1-5 MWh is considered medium, and an enterprise with greater than 5 MWh usage is large.

This criterion divides the total consumers into three groups evenly, meaning each group has a similar and fair impact on the average values. In different cases, the specific criterion depends on the specific companies and the distribution of different usage quantities on the scale.

Additionally, the consumers can be categorized into different sensitive levels to the electricity price, R_i , according to the following equation:

$$R_i = \text{annual electricity expenditure} / \text{annual revenue} * 100\%$$

Highly sensitive enterprises have $R_i \geq 4\%$, medium sensitive enterprises have $1\% \leq R_i \leq 4\%$, and low sensitive enterprises have $R_i < 1\%$

Based on the sizes of different consumers and different sensitivities, the researchers developed three other variables, k_i , $risk_i$, and σ_i to reflect the heterogeneity's change to the original rates of the SIR model. k_i reflects the rationality of consumer

i , measuring how readily the consumer's decision is affected by the social environment. Xianjia Wang, Culing Gu, Jinhua Zhao, and Ji Quan use the Fermi function to explain the impact of k on an individual's decision-making: when $0 \leq k < 1$, the individual is perfectly rational; as the k value increases till 20, the individual tends to rely more on their environment.⁸ Therefore, k base equals 8, implying typical human behavior - neutral rationality - in decision-making. Then, k is multiplied by different coefficients to represent the behavioral tendency according to the sensitivity of different consumers. $risk_i$ is the risk reversion of consumers, in other words, how likely they are to end their collaboration with the VPP due to potential risk. The risk base is set to 1, referencing the beta, which measures investment volatility in financial economics, a beta of 1 serves as a benchmark for enterprises that vary exactly with market swings. A beta greater than 1 indicates enterprises that are more volatile than the market, while a beta less than 1 indicates less volatile ones. Therefore, in the case of virtual power plants, the risk base is multiplied by different coefficients based on size, making it equal to, less than, or greater than 1: large consumers are willing and able to take losses, while smaller consumers are more sensitive. Larger consumers have a smaller $risk_i$, meaning they are less responsive and volatile to market risks, while smaller consumers are the opposite. σ_i models consumers' decisions' impact on society. In order to represent different levels of impact by different sizes of consumers, a σ base is needed. Tverskoi, Badu, and Gavrilits analyze the significant impact of the public's acceptance of a technology on the spreading of such technology, and model conformity of peers, which is a key factor in an individual's acceptance, into an S-shaped diffusion curve.⁹ According to Tverskoi and his peers, the S-shaped diffusion curve is commonly shown when modeling problems that involve the spread of social behaviors. For a clear and realistic S-shaped diffusion curve of collaboration, the base value is set to 1, multiplied by different coefficients based on the consumer's size: larger enterprises' decision-making processes are usually private and independent, whereas small enterprises' results are more easily observed and mimicked by other enterprises in society. All the multipliers used in this section are illustrative, but reasonable parameters to quantify and simulate the heterogeneity among consumers. Through algorithm experimentation, the model's results are stable and reliable within the range of $\pm 15\%$ to $\pm 35\%$ of the illustrative multipliers. Based on their size and sensitivity, enterprises are divided into 9 categories, summarized in Table 1.

Table 1: Categories of enterprises. Categorize diverse consumers into 9 groups with corresponding rationality, risk aversion, and impact level based on size and sensitivity to price change.

Electricity Sensitivity	Size	Rationality k_i	Risk Aversion $risk_i$	Impact on society σ_i
High	Large	8*1.25	1*0.8	1*0.7
High	Medium	8*1.25	1*1.0	1*1.0
High	Small	8*1.25	1*1.3	1*1.3
Medium	Large	8*1.0	1*0.8	1*0.7
Medium	Medium	8*1.0	1*1.0	1*1.0
Medium	Small	8*1.0	1*1.3	1*1.3
Low	Large	8*0.75	1*0.8	1*0.7
Low	Medium	8*0.75	1*1.0	1*1.0
Low	Small	8*0.75	1*1.3	1*1.3

- Development of the dynamic SIR model:

The Basic SIR model is the following:

$$\begin{aligned}\frac{dS}{dt} &= -\beta(m) \cdot I(m) \cdot \frac{S(m)}{N} + \delta(m) \cdot R(m) \\ \frac{dI}{dt} &= \beta(m) \cdot I(m) \cdot \frac{S(m)}{N} + \gamma(m) \cdot I(m) \\ \frac{dR}{dt} &= \gamma(m) \cdot I(m) - \delta(m) \cdot R(m)\end{aligned}$$

Specifically, the infection rate, $\beta(m)$, is the monthly rate that potential collaborators become real collaborators; the recovery, $\gamma(m)$, is the monthly rate that collaborators exit collaboration; and the reinfection rate, $\delta(m)$, is the monthly rate that recovered enterprise reenters collaboration. N , $S(m)$, $I(m)$, and $R(m)$ are the monthly total population, susceptible population, infected population, and recovered population. Below are the specific steps to change those constant rates into dynamic rates to better illustrate the inter-enterprise influence and real-time changes in corporate decisions.

a. Dynamic $\beta(m)$:

Researchers utilize the Fermi Function to illustrate how an enterprise's decision to join or not depends on its perception of profit difference. The Fermi Function is a common rule used in Evolutionary Game Theory, a model of individuals interacting through strategic games, where success depends on how widespread their strategies are.¹⁰ The advantage of such game theory is that it allows individuals to adjust their strategies in response to environmental changes; in other words, it does not assume that all individuals are rational.⁸ In the case of VPP, the strategies discussed are whether to join the collaboration, where joined enterprises can influence others' strategies and turn them into collaboration. Specifically, the Fermi Function explains the linear relationship between profit difference and the probability of individuals taking certain strategies.⁸ Therefore, the Fermi Function is a great tool to define $\beta(m)$, the spread of collaboration. The basic formula of the Fermi Function is below:¹⁰

$$p_f(\pi, \pi') = \frac{1}{(1 + e^{-s(\pi' - \pi)})}$$

$p_f(\pi, \pi')$ is the probability for individuals to change their decision from π to π' based on the profit difference $(\pi' - \pi)$ and weights.

Therefore, the probability for individual consumer i to change from non-cooperation to cooperation can be written as:

$$G_i(\Delta\pi_i(m)) = \frac{1}{1 + e^{-k_i \cdot \Delta\pi_i(m)}}$$

k_i is the rationality of consumer i , meaning how easily influenced the consumer is by changes in incentives. $\Delta\pi_i$ is the profit difference, defined $incentive_j(m) \cdot p_i - c_i^{adj}$, where $incentive_j(m)$ is the incentive provided that month, p_i is the adjustable power, and c_i^{adj} is the collaboration cost.

Then, calculate the weight average of everyone's collaboration rate to obtain $\beta(m)$:

$$\beta(m) = \frac{(\sum_{i=1}^N [w_i \cdot G_i(\Delta\pi_i(m))])}{N}$$

$w_i = \frac{(\rho_i \cdot \sigma_i)}{\sum_{i=1}^N (\rho_i \cdot \sigma_i)}$, representing the level of impact of consumer i 's decision on society.

b. Dynamic $\gamma(m)$

The basic definition of $\gamma(m)$ can be written as:

$$\gamma(m) = \gamma_{0,i} \cdot \eta_{risk,i} \cdot \eta_{profit,i}(m)$$

$\gamma_{0,i}$ is the basic exit rate that occurs in human natural tendency: according to Reinartz and Kumar, businesses cannot decrease their cooperation exit rate to zero simply by adjusting operation strategies; therefore, there is a natural, inherent exit rate.¹¹ The exact value of $\gamma_{0,i}$ which is 0.02/month, is calculated from enterprises' account retention over years, including Ameritrade and Capital One, in the study by Sunil Gupta, Donald Lehmann, and Jennifer Stuart, and verified by the customer turnover of the cellular telephone industry, which 2.7% per month, in the study by Roth Bolton.^{12,13} $\eta_{risk,i}$ is the risk factor, representing consumers' exit from collaboration due to risk aversion. $\eta_{profit,i}$ is the profit factor, representing consumers' exit from collaboration due to the failure to realize their ideal profit.

The definition of profit factor is based on the prospect theory, which is shown below:

$$V(x, p; y, q) = v(y) + \pi(p)[v(x) - v(y)]$$

, which includes choice x and y and their outcome, respectively, p and q . $\pi(p)$ is the weight, showing how sensitive individuals are to failure in profit. $v(x) - v(y)$ is the comparison between expected profit and actual profit.

Therefore, in the VPP scenario, the prospect theory can be applied as follows:

$$\eta_{profit,i}(m) = 1 + \alpha \cdot \max(0, \Delta\pi_{threshold} - \Delta\pi_i(m))$$

$\Delta\pi_{threshold}$ is how much profit the consumer i expects to gain by collaborating, and is set to be $1.2^* c_i^{adj}$ because consumers expect to obtain basic profit outside of the cost; $\Delta\pi_i(m)$ is the same as in section a. α is set to be 0.5 to illustrate a clear SIR curve, avoiding excessively high attrition rates leading to the collapse of the collaborative group.

The definition of the risk factor is based on the linear relationship between the consumers' expected profit and the level of risk, shown by the Capital Asset Pricing Model. Therefore, the risk level has a linear effect on the $\gamma(m)$:

$$\eta_{risk} = 1 + \alpha_{risk} \cdot risk_i$$

α_{risk} is the weight that shows the level of Impact of consumers' inherent risk aversion on their exit decisions. To ensure the model produces results that match empirical observations and theoretical expectations, the exit rate of high-risk-aversion consumers should be higher than that of low-risk-aversion consumers. Therefore, after calibrating using the corresponding risk value α_{risk} , is set to 0.56.

c. Dynamic $\delta(m)$:

The definition of $\delta(m)$ is:

$$\delta(m) = \delta_0 + \alpha_{reinfect} \cdot \frac{I(m)}{N}$$

δ_0 is the basic return rate, representing naturally re-entering collaboration due to memory decay and favoring reciprocity

without external influence. It is set to be 0.01/month based on studies on natural retention rate in customer relationship management [14]. $\alpha_{reinflect} \cdot \frac{I(m)}{N}$ shows the attraction that other consumers' collaboration has on withdrawn consumers. $\alpha_{reinflect}$ quantifies such an effect; according to Ash's study on conformity behavior in social psychology, the value of $\alpha_{reinflect}$ is set to be 0.01.¹⁵

- A bilevel model between consumer collaboration and VPP profit:

Bilevel optimization models simulate decision-making in hierarchical settings and are widely used by public and private organizations in large-scale planning. The model consists of an upper level, the leader, which proposes solutions to optimize its goals, and a lower level, the follower, which responds to every proposed solution and reacts accordingly. The reaction of the lower layer will affect the final result of the upper layer's optimization.

a. Upper optimization level — VPP's profit maximization:

VPP acts as a price-maker, providing incentives to increase the consumer base and maximize profits.

$$\max \text{profit} = \sum_{m=1}^{12} \left[\sum_{i \in I(m)} p_i \cdot (\lambda_{\text{market}}(m) - \text{incentive}_i(m) - b_{op}(m)) \right]$$

$\lambda_{\text{market}}(m)$ is the market electricity price, $\text{incentive}_i(m)$ is the proposed incentives, $b_{op}(m)$ is the operation cost.

b. Lower level — SIR model:

The definition of the SIR model and its rates are the same as in the section Development of the dynamic SIR model. After the initial state of the cooperative group is set, the proposed incentive values will be input into the SIR model, which will report back the corresponding $I(m)$ and p_i values to the upper level. To quantify such an effect, whose value is set to be 0.01.

Solution to the proposed bilevel model:

The researchers use Python to call the HPSO-SA (Hybrid Particle Swarm Optimization - Simulated Annealing) algorithm, which can effectively solve the proposed complex bilevel problem.

PSO, particle swarm optimization (PSO), is a commonly used method to solve bilevel models that deal with a single decision maker and a follower.¹⁶ Both the leader and follower react optimally, where the follower has output y made based on the leader's solution x . PSO generates an initial population of random solutions and iteratively updates them to search for the optimal solution.¹⁶ The optimal solution is identified by its optimal fitness, which corresponds to the objective function value. Solutions are represented by particles flying in the space and move with the current optimal particles. Particles adjust their trajectory according to two references: the best position in their own history, p_{best} , and the current best position in the swarm, g_{best} . Each time PSO locates a better particle, the p_{best} and g_{best} will update accordingly. The updating rule for each particle is the following:¹⁶

$$\begin{aligned} \text{Updated velocity} : V_{\text{new}} &= wV_{\text{old}} + c_1 r_1 (p_{\text{best}} - X_{\text{old}}) + c_2 r_2 (g_{\text{best}} - X_{\text{old}}) \\ \text{Updated velocity} : X_{\text{new}} &= X_{\text{old}} + V_{\text{new}} \end{aligned}$$

Above, w represents the inertia weight, c_1 and c_2 are learning factors for cognitive and social influence, respectively, and r_1 and r_2 are two random numbers evenly distributed in $[0,1]$. After updates, PSO will run through another iteration and repeat the process until it reaches the maximum number of iterations and minimal improvement.

The proposed model has great complexity: first of all, it is a classical bilevel model where the upper level's incentive is closely connected to the lower level's cooperation rate; secondly, the heterogeneity within the consumer base causes effective incentives to be different for different consumers; thirdly, given the market uncertainty, the VPP should adjust incentive prices in real time. Therefore, the variables in the optimization problems have 72 dimensions:

$$\text{DecisionVariableDimensions} = 3(\text{categories}) \cdot 24(\text{months}) = 72\text{Dimensions}$$

In such a high-dimensional decision space, conventional PSO can hardly solve the problem effectively. Therefore, the researchers developed an improved PSO.

Basic PSO can easily get stuck in local optimals and affect the accuracy of the final outcome. Therefore, researchers integrate simulated annealing (SA) into PSO to occasionally accept worse particles, potential candidates for g_{best} , and compare them with the optimal g_{best} particle. If candidates have better fitness than the original g_{best} , the new g_{best} is calculated based on the superior candidate. If the candidates have worse fitness, accept it as the new g_{best} with probability:¹⁷

$$P = e^{-\frac{\Delta}{T}}$$

, where Δ is the fitness difference, and T is the current cooling temperature.

In addition, to illustrate the dynamic exploration of PSO, researchers process w , c_1 , and c_2 into three linear equations:

$$\begin{aligned} w &= w_{\text{max}} - \frac{(w_{\text{max}} - w_{\text{min}}) \cdot k}{K_{\text{max}}} \\ c_1 &= c_{1\text{max}} - \frac{((c_{1\text{max}} - c_{1\text{min}}) \cdot k)}{K_{\text{max}}} \\ c_2 &= c_{2\text{min}} + \frac{((c_{2\text{max}} - c_{2\text{min}}) \cdot k)}{K_{\text{max}}} \end{aligned}$$

, where k is the iteration number, and K_{max} is the maximum iteration number. In this way, at the first few iterations, larger w promotes particles to explore a broader region, and larger c_1 encourages particles to broadly search for a solution and avoid early convergence; as the iterations process, smaller w allows particles to explore more keenly and precisely in the optimal region, and larger c_2 enables the swarm to quickly narrow down to the best solutions areas for high precision.

Therefore, according to the design above, the HPSO-SA solution will run the lower-level SIR model, output the fitness of each particle (representing different incentives), update g_{best} and p_{best} , and each particle's position and velocity, and adjust the inertia weight and learning factors. Meanwhile, apply simulated annealing to the g_{best} particle to refine it, freeing the solution from local optima. Finally, when maximum iterations are reached and minimal improvements are made, output the optimal incentives and SIR curves, visualizing the calculation results.

In the VPP profit-maximization scenario, the profit-maximization function provides random incentives (particles) as input to the lower SIR model.

Below is the definition of fitness:

$$F_{\text{fitness}} = \text{Profit}_{\text{total}} - n_1 \cdot \max(0, CR_{\text{target}} - CR_{\text{final}})^3 + n_2 \cdot CR_{\text{avg}} - n_3 \cdot \sigma_{CR}$$

This equation uses the upper-level profit function as its core and integrates penalty and bonus terms to ensure that both profit and the cooperation ratio (I/N) are optimal and stable. Within the function, CR_{target} is the targeted cooperation ratio, CR_{final} is the total cooperation ratio at the end of the time range, σ_{CR} is the standard deviation that measures the stability of cooperation. n_1 , n_2 , and n_3 are all weighting coefficients.

Case Study:

a. Consumer base and heterogeneous categorization:

To demonstrate the proposed model's availability, researchers implemented it using market research data from 500 representative industrial enterprises, which were chosen randomly to illustrate the method's universality, aggregated by a virtual power plant in a city in southwest China. Researchers divided those 500 enterprises into 3 categories according to the categorization rule provided in the Model Derivation section, shown in the table below.

Table 2: Example enterprise categories. Based on the size and sensitivity, the sample consumers can be divided into 3 groups.

Categories	Electricity price sensitivity	Size	Customers population	Electricity consumption	Rationality k_e	Risk Reversion risk	Impact on society α
1	High	Medium	231	13.7	10.0	1.0	1.0
2	Medium	Small	129	12.7	8.0	1.3	1.3
3	Low	Large	140	14.2	6.0	0.8	0.7

b. PSO-SA Parameter Settings:

As mentioned earlier, this model is a relatively difficult bi-level optimization problem with 72 dimensions; therefore, the particle population is set to be larger than that of typical bi-level problems, which are usually 20-40. Other parameters for the PSO algorithm are based on He, Ma, and Zhang's studies on the influence of parameter settings on PSO performance, whereas the parameters of simulated annealing are based on the study by Park and Kim.^{18,19}

$N_{\text{pop}}=400$, $K_{\text{max}}=250$, $W_{\text{max}}=[0.4, 0.9]$, $c_1=c_2=2$, $T_{\text{initial}}=100$, attenuation coefficient $\alpha_{SA}=0.95$, bound = $[0.00035, 0.0045]$, $\text{velocity}_{\text{max}}=0.15$

c. Initial cooperation state settings:

$S = 400$, $I = 80$, $R = 20$

d. Net profit per month without incentives (Table 3):

The net profit is obtained through market research on the virtual power plant operator in western China.

$$\text{Net Profit} = \lambda_{\text{market}}(m) - b_{\text{op}}(m)$$

Table 3: Net profit each month. The monthly net profits reflect the southwest area's reliance on hydropower generation, where months 1, 2, 3, 4, 5, 13, 14, 15, 16, 17 are high-water season with great electricity profit, months 7, 8, 9, 10, 11, 19, 20, 21, 22, 23 are low-water season with low electricity profit, and month 6, 12, 18, 24 are transition period in between.

Month	1	2	3	4	5	6	7	8	9	10	11	12
Net Profit (yuan/kWh)	0.0085	0.0092	0.0096	0.0088	0.009	0.0062	0.0012	0.001	0.0012	0.001	0.0008	0.0065
Month	13	14	15	16	17	18	19	20	21	22	23	24
Net profit (yuan/kWh)	0.0085	0.0092	0.0096	0.0088	0.009	0.0062	0.0012	0.001	0.0012	0.001	0.0008	0.0065

e. Comparative Experiment:

In order to demonstrate the superiority of the designed model, the researchers use three methods to create the best incentive strategy for 2 years.

Method 1: Conventional operation of the VPP, where the VPP does not provide incentives to consumers.

Method 2: Propose fixed incentives for each category throughout the 2-year range by employing optimization algorithms.

Method 3: Uses the proposed bilevel model above, which incorporates dynamic incentives for different categories over time.

Results and Discussion

Comparison Between Three Methods:

Based on Figure 2, the third method clearly shows superiority in both the cooperation rate (CR) and profit. First of all, the result shows the necessity of applying incentives as both the second and the third methods surpass the targeted CR, 80%, while the CR of the first method is only 65.5%. This shows that giving consumers incentives is an effective way to solve cooperation problems. Additionally, comparing method 2 and method 3, the researchers conclude that the dynamic incentive is better for the balance between cooperation rate and VPP's profit: provided that the cooperation rate does not decline, dynamic incentives yield the highest profits. Therefore, even though incentives can effectively stabilize the consumer base, a fixed, rigid strategy can devour profits to maintain a high cooperation rate, whereas a flexible strategy ensures both by spatiotemporally refining the allocation of incentive budgets.

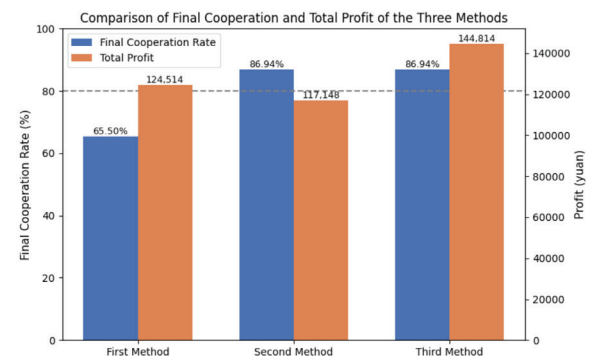


Figure 2: Comparison of final cooperations and total profits of the three methods. The third method reaches the highest profit and best balance between profit and cooperation.

Detailed Analysis of the Third Method:

After the PSO-SA calculation of the model designed through Python, the target VPP exhibits significant financial profit and synergy. First of all, based on Figure 3, VPP exhibits a great and stable participation rate with an average of 87%, which is successfully higher than the target rate 80%. This shows the positive effect of such an incentive strategy on the VPP's consumer base. In addition, consumers in different categories exhibit different participation rates, as shown in Figure 4. The larger size consumer (High) has significantly higher participation, while the Small size consumer (Low) and the Medium size consumers (Med) have similar participation.

The differences in participation are due to differences in the electricity consumption scale and the sensitivity to incentives: Large consumers have a larger scale and lower sensitivity, and therefore exhibit a higher and more stable participation rate; even though small consumers have a smaller sensitivity than the medium-sized consumers, they have a lot less energy consumption and population, which explains why they have a slightly lower participation rate and a higher amount of average incentive in Figure 5. This shows the necessity of including consumer heterogeneity in the model, which guides the VPP in offering different and effective incentives shown in Figure 7. The result also shows how the model is designed to create adaptive and granular budgeting strategies for VPPs. Based on Figure 3, the average total incentives are highest at months 14 and 15, which have the highest next profit. The model, therefore, shows how VPP should appropriately increase the amount of incentives when the market price increases to balance its profit and the participation rate. The strategy creates economic profit for both the VPP and consumers: under such an incentive system, the consumers are able to save 0.0037 ¥/MW, and the total profit generated by the VPP is ¥144,814.23 based on Figure 6.

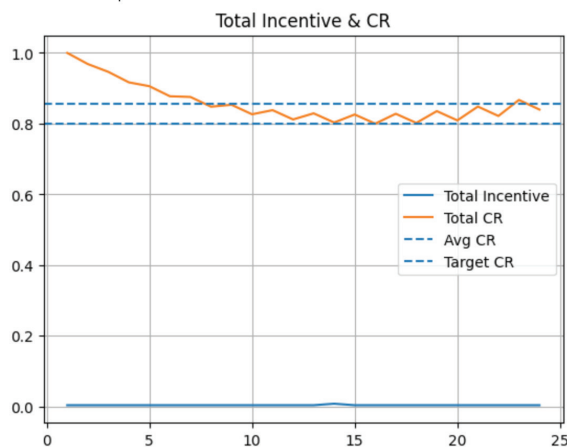


Figure 3: Total cooperation rates (CR) and total incentives per month. Total cooperation rate remains above target throughout the studied time range, with a higher cooperation rate and incentive during the high-water season.

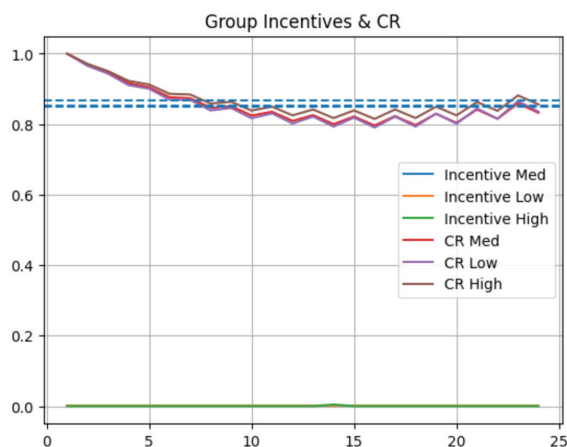


Figure 4: Categorical participation rates (CR) and categorical incentive amount (MW/yuan). The cooperation rate of big-sized consumers exhibits a significantly larger cooperation rate than that of smaller consumers.

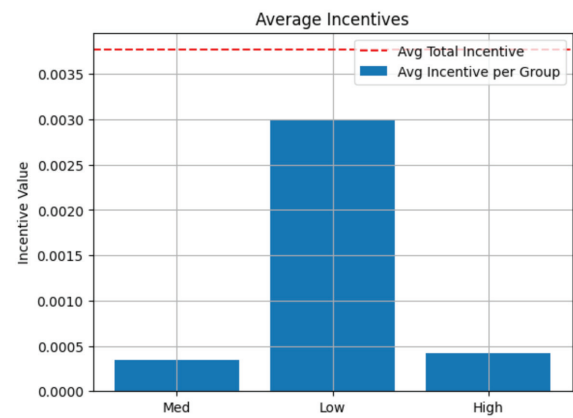


Figure 5: Enlarged version of total and categorical incentive amount (MW/yuan). Small-sized consumers require a higher incentive compared to bigger consumers.

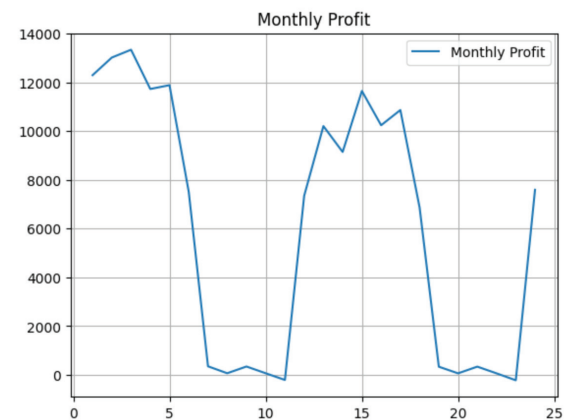


Figure 6: Monthly profit of the virtual power plant. Monthly total profit corresponds to the profit differences between high-water season and low-water season and sums up to a positive annual profit.

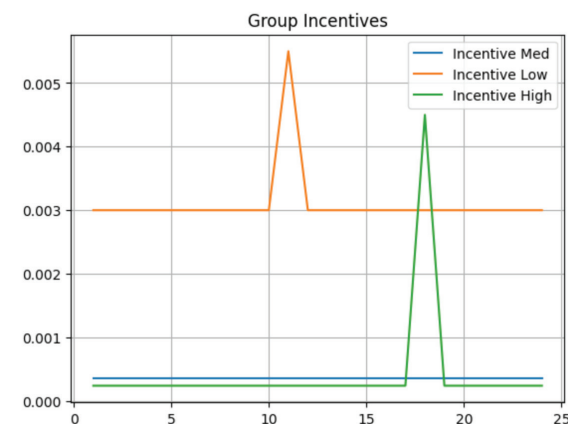


Figure 7: Monthly incentives for each category. The incentive strategy unevenly allocates incentives among heterogeneous consumer groups to meet the requirements of diverse consumers in a personalized manner.

Conclusion

Based on the result above, the proposed bilevel system successfully developed an incentive strategy for VPP's long-term profitability and stability of cooperative relationships. The strategy also emphasizes heterogeneity within the consumer base and responds with different incentive amounts, a factor largely overlooked in previous studies. The modified PSO-SA

algorithm serves to accurately solve such complicated models and provide robust technical support. Therefore, the model provides not only a deep and novel understanding of coordinating distributed resources, but also an actionable and insightful practical guide for VPP's operation under modern electricity systems. However, research on VPP market pricing extends far beyond this point. Future research can effectively increase the models' accuracy by specifically improving the categorization system in order to incorporate more diverse consumers and track subtle interactions between them. The multipliers used in categorification also vary depending on the specific virtual power plant consumer groups: the proposed model is proven to be viable, yet real applications need calibration. Additionally, specific methods to implement different incentives remain vague: future studies are needed in order to avoid customer disputes resulting from price discrimination between different categories.

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Financial Literacy Knowledge and Practices among Chinese Adolescents: A Survey-Based Analysis

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ABSTRACT: Financial literacy is increasingly recognized as a critical life skill that lays the foundation for rational financial decision-making, healthy consumption habits, and long-term economic well-being. However, systematic research on the financial literacy level, related knowledge mastery, and practical behaviors of Chinese adolescents remains limited, particularly from the perspective of high school researchers. This study examined the financial literacy knowledge, attitudes, and practices of Chinese middle and high school students through a quantitative questionnaire survey design. A total of 200 respondents from the Beijing Academy participated voluntarily and anonymously, with 158 valid questionnaires analyzed via descriptive statistical analysis. Results indicate that most Chinese junior and senior high school students have basic financial awareness (such as recognizing common currencies and simple consumption concepts) but lack systematic financial knowledge and standardized financial practices. The level of financial literacy varies with grade, family education background, and school financial education resources. Key challenges include insufficient school-based financial education courses, limited family financial communication, and low awareness of rational consumption, budgeting, and risk prevention among adolescents. This study highlights the urgency of integrating systematic financial education into junior and senior high school curricula and provides practical implications for educators, parents, and policymakers to improve Chinese adolescents' financial literacy.

KEYWORDS: Economics, Financial Literacy, Adolescents, Financial Education, Financial Behavior.

■ Introduction

In the context of increasing economic globalization and diversified consumption patterns, financial literacy has become an essential quality for individuals to adapt to social life. Financial literacy refers to the ability of individuals to master basic financial knowledge, understand financial products and risks, and make rational financial decisions and standardized financial behaviors to meet their own economic needs and achieve long-term financial well-being.¹ For adolescents who are in the critical period of values formation and behavioral habits cultivation, good financial literacy not only helps them avoid irrational consumption and overspending in daily life but also lays a solid foundation for their independent financial management ability after adulthood.²

In recent years, with the popularization of digital payments and the improvement of family living standards, Chinese junior and senior high school students have more opportunities to contact and use financial products (such as pocket money, red envelopes, and electronic payment tools), and their financial behaviors have become increasingly frequent. However, compared with the growing demand for financial literacy, the current financial education for Chinese junior and senior high school students is still in the initial stage: most schools do not set up specialized financial education courses, and financial knowledge is only occasionally involved in subjects such as politics, morality, and society.³ Family financial education is also uneven, and some parents pay little attention to guiding their children to establish correct consumption concepts and financial awareness.^{4,5} As a result, many junior and senior

high school students have misunderstandings about financial knowledge, lack the ability to budget and manage money, and even develop problematic financial behaviors such as excessive consumption and comparison consumption.

At present, most existing studies on adolescent financial literacy in China are carried out by scholars or researchers, and there are few studies completed independently by high school students from the perspective of peers. As a high school student who pays attention to adolescent growth and financial education, this study aims to fill this small research gap through a practical investigation. This study not only meets the requirements for original student research but also provides a reference for improving the financial education level of Chinese junior and senior high schools and promoting the healthy growth of adolescents.

Based on the above background, this study focuses on the following research questions: 1) What is the current level of financial literacy (financial knowledge, financial attitudes, financial behaviors) among Chinese junior and senior high school students? 2) What are the main problems existing in junior and senior high school students' financial literacy and financial practices? 3) What factors affect the formation of junior and senior high school students' financial literacy, and how can it be improved? To address these questions, a cross-sectional questionnaire survey was conducted among students of Beijing Academy, covering grades 7 to 12, with descriptive statistical analysis adopted to process and interpret the collected data.

■ Methods

This study employed a quantitative, cross-sectional survey design to assess the financial literacy knowledge, attitudes, and behaviors of Chinese adolescents. The research was conducted among students at Beijing Academy, in Beijing, P.R. China.

Participants and Sampling:

A total of 200 students from grades 7 to 12 were invited to participate on a voluntary and anonymous basis. The sampling strategy aimed for gender balance and stratification by grade level (junior high: grades 7–9; senior high: grades 10–12). After excluding incomplete or inconsistent responses, 158 questionnaires were retained for analysis, yielding an effective response rate of 79%.

Instrument and Measures:

Data were collected using a self-administered, structured questionnaire developed by the author. The instrument was designed based on established financial literacy frameworks from international assessments such as PISA⁶ and national studies on youth finance.^{1,3} It was then adapted to reflect the Chinese adolescent context, including familiarity with digital payment platforms like WeChat Pay and Alipay. The final questionnaire comprised four sections:

- (1) Demographic information (e.g., grade, gender, family income, frequency of family financial communication);
- (2) Financial knowledge, assessed through multiple-choice questions on core concepts such as interest, inflation, budgeting, and risk diversification;
- (3) Financial attitudes, measured using 5-point Likert-scale items (1 = Strongly Disagree to 5 = Strongly Agree) regarding saving habits, rational consumption, and peer influence;
- (4) Self-reported financial behaviors, including daily spending patterns, saving practices, use of budgets, and experiences with impulsive purchases.

The questionnaire underwent a pilot test with 10 students to ensure clarity and face validity prior to full deployment.

Data Analysis:

All data were analyzed using descriptive statistics. Frequencies and percentages were calculated to summarize sample characteristics and to describe levels of financial knowledge, attitudes, and behaviors. Analyses were performed using Microsoft Excel to provide an accessible and transparent overview of the current state of adolescent financial literacy in the sample.

■ Results and Discussion

Demographic Information of Respondents:

A total of 158 valid questionnaires were obtained from the 200 distributed to students of Beijing Academy (grades 7–12), with an effective recovery rate of 79%. The demographic characteristics of the respondents are presented in Table 1, which provides a detailed overview of the sample composition across five key dimensions: school level (junior vs. senior high), gender, family income level, frequency of family financial communication, and availability of school-based financial education. As

shown in Table 1, the sample achieves balanced representation in terms of gender (50% male, 50% female) and includes both junior high (40%) and senior high (60%) students, ensuring relevance across the adolescent age spectrum studied.

Table 1: Demographic characteristics of the 158 valid survey respondents, showing the proportional distribution (%) across core sociodemographic and educational variables. The table highlights the prevalence of middle-or-higher family income (50%), infrequent family financial discussions (70% report "no/rare" communication), and the striking absence of formal financial education in schools (90% report it as unavailable). These contextual factors are critical for interpreting the financial literacy outcomes reported in subsequent sections.

Characteristic	Category	Proportion (%)
Grade	Junior high (7–9)	40
	Senior high (10–12)	60
Gender	Male	50
	Female	50
Family income	Middle or higher	50
	Others	50
Family financial communication	Regular	30
	No/rare	70
School financial education	Available	10
	Unavailable	90

The data in Table 1 reveal two systemic deficiencies in the financial literacy support environment for Chinese adolescents. First, despite half of the respondents coming from families with middle or higher income, only 30% report having regular conversations about money, saving, or budgeting with their guardians—indicating a widespread gap in informal financial socialization at home. Second, and more critically, a vast majority (90%) of students attend schools where no dedicated financial education is provided, confirming that institutional support for financial learning remains minimal in the current curriculum framework. These structural limitations help explain why, as detailed below, many students demonstrate only superficial financial understanding and inconsistent money management behaviors, despite possessing basic awareness of consumption and saving.

This demographic profile ensures the basic representativeness of the sample for the target population and establishes a clear foundation for analyzing how family background and school resources shape financial literacy development among Chinese adolescents.

Current Situation of Adolescents' Financial Literacy Knowledge:

The survey shows that Chinese adolescents have a certain level of basic financial knowledge but lack systematic and in-depth financial knowledge. Specifically, 90% of the respondents can correctly recognize common RMB denominations and understand the basic meaning of "saving", and 80% can distinguish between "income" and "expenditure". However, for more professional financial knowledge, such as the concept of interest, inflation, financial risks, and financial products (such as savings, funds), the mastery rate of adolescents is relatively low: only 50% of the respondents can correctly explain the concept of interest, 30% understand the meaning of inflation, and less than 20% know about common financial products. In addition, there are significant differences in the mastery of financial knowledge among adolescents of different grades: the

higher the grade, the better the mastery of financial knowledge, which is related to the improvement of their cognitive level and the accumulation of life experience. This finding is consistent with the cognitive development law of adolescents, reflecting that financial knowledge acquisition is a gradual process accompanied by age and learning experience.

Current Situation of Adolescents' Financial Attitudes:

Most Chinese adolescents hold a relatively rational attitude towards money and consumption, but there are still some incorrect attitudes that may lead to problematic financial behaviors. Seventy percent of the respondents stated that "money should be spent reasonably" and "saving is a good habit", and 50% said that they would not spend money beyond their means. However, 35% of the respondents hold the attitude of "enjoying now" and are willing to spend money on favorite things even if they do not need them, and 20% have a psychology of comparison, believing that "spending more money can show their ability or status". In addition, the financial attitudes of adolescents are significantly affected by family education: adolescents from families with good financial communication and positive guidance have more rational financial attitudes, while adolescents from families that dote on their children or pay little attention to financial guidance are more likely to have incorrect financial attitudes. This result verifies the key role of family in the formation of adolescent financial values, as parents' financial concepts and educational methods have a profound and direct impact on their children's attitude towards money.

Current Situation of Adolescents' Financial Behaviors:

The financial behaviors of Chinese adolescents are mainly concentrated on daily consumption and simple saving, and the overall level of standardized financial management is relatively low. Fifty percent of the respondents have the habit of saving pocket money or red envelope money, but most of them save money just to buy their favorite things (such as toys, books, and electronic products) rather than for long-term financial planning. Eighty percent of the respondents' daily consumption is mainly used for food, stationery, and entertainment, and a few respondents have the behavior of budgeting, recording expenses, or attempting basic investment. In addition, 30% of the respondents have experienced overspending, mainly due to impulsive consumption or comparison consumption triggered by the aforementioned incorrect financial attitudes. Notably, 85% of the respondents have used electronic payment tools (such as WeChat Pay, Alipay), which is in line with the popularization of digital payment in China, but they lack corresponding risk prevention awareness and do not pay attention to protecting their personal financial information, posing potential financial risks for adolescents.

Interpretation of Research Results:

The research finds that Chinese junior and senior high school students have basic financial awareness and relatively rational financial attitudes on the whole, but there are obvious deficiencies in professional financial knowledge mastery and

standardized financial practice ability, with the overall financial literacy level at a medium level, which is consistent with the research conclusions of most domestic scholars on adolescent financial literacy.³ The advantages of Chinese junior and senior high school students' financial literacy are reflected in their basic recognition of money and rational consumption attitudes, which are closely related to the traditional virtue of "thrift" in Chinese culture and the basic moral education carried out in schools. However, the deficiencies are more prominent and multi-faceted: first, the lack of systematic financial knowledge, which is mainly due to the absence of specialized financial education courses in junior and senior high schools, making it impossible for adolescents to form a complete and systematic financial knowledge system; second, the lack of standardized financial practices, with most students not having the habits of budgeting, recording expenses, and long-term saving, resulting in weak independent financial management ability; third, some students have incorrect financial attitudes (such as comparison consumption, impulsive consumption), which are jointly affected by family education, social environment, and media publicity.

In addition, the research confirms that the financial literacy level of junior and senior high school students is significantly affected by three key factors: grade, family education background, and school financial education resources, the distribution of which is detailed in Table 1. The higher the grade, the better the financial literacy performance, because with the growth of age and the development of cognitive ability, students can accumulate more financial knowledge and practical experience through daily life and learning. Adolescents from families with good financial communication and positive guidance have better financial literacy, indicating that family is an important place for adolescent financial education, and parents' intentional guidance plays a key role in the formation of students' financial literacy.⁴ Schools that integrate financial education content into daily teaching can effectively help students master basic financial knowledge, showing that school-based financial education is an important and effective way to improve adolescent financial literacy.

Comparison with Existing Studies:

Compared with existing domestic and international studies on adolescent financial literacy, this study has two distinctive characteristics: first, from the research perspective, this study is completed independently by a high school student, with a more intuitive and in-depth understanding of the actual financial status and real needs of peers, and the research content is closer to the daily life of Chinese junior and senior high school students;^{1, 2} second, from the research object, this study focuses on Chinese junior and senior high school students covering grades 7 to 12, with a balanced gender distribution and stratified grade sampling as shown in Table 1, which provides a representative sample of Chinese adolescents in the same age group.

The research results are basically consistent with the conclusions of existing domestic and international studies, such as the common finding that adolescent financial literacy is

comprehensively affected by family, school, and individual factors, and the lack of systematic school financial education is an important reason for the low level of students' financial literacy.³⁻⁵ At the same time, this study also finds that the financial practice ability of Chinese junior and senior high school students is obviously weaker than that of adolescents in some developed countries, which is closely related to the late start of financial education in China and the insufficient attention paid to the training of practical financial management ability in the current education system. Most developed countries have integrated financial education into the compulsory education curriculum for a long time and pay attention to the combination of theoretical knowledge and practical operation, which is a valuable experience that China can learn from in the construction of the adolescent financial education system.

Analysis of Influencing Factors:

Combined with the research results and the actual situation of Chinese adolescent education, the main factors affecting the financial literacy and financial behavior of Chinese junior and senior high school students can be summarized into three aspects: school, family, and society, which interact and jointly shape the financial literacy level of adolescents.

First, school factors are the core institutional factors affecting adolescent financial literacy. Currently, most secondary schools in China do not offer dedicated financial education courses, and financial literacy content is only sporadically covered in other subjects in a fragmented manner, such as politics and morality, which cannot meet the actual needs of students for financial literacy improvement.³ In addition, most teachers in the basic education stage lack professional financial education training and systematic financial knowledge reserves, and cannot provide professional and systematic financial guidance for students, resulting in the failure of school financial education to play its due role.

Second, family factors are the most direct and fundamental factor affecting adolescent financial literacy. Family is the first place where junior and senior high school students come into contact with money and carry out financial behaviors, and parents' financial concepts, consumption habits, and educational methods have a profound and lasting impact on the formation of their children's financial literacy.⁴ Some parents dote on their children, meet all their material needs unconditionally, and do not guide them to establish correct consumption concepts and the awareness of "hard-won money"; some parents pay little attention to financial communication with their children, and do not intentionally cultivate their children's ability of independent financial management such as budgeting and saving, leading to the lack of practical experience of financial management among adolescents.

Third, social factors are an important external factor affecting adolescent financial literacy. With the popularization of digital media and the rise of consumer culture, some problematic consumption concepts (such as hedonism, comparison consumption, and excessive consumption) have spread widely in society and have a negative impact on the values of junior and senior high school students who are in the critical period

of values formation. At the same time, the social financial education system in China is not yet perfect, and there are few targeted and systematic financial education activities and resources for adolescents. In addition, the supervision of financial products and consumer markets related to adolescents is not strict enough, which also makes it easy for adolescents to be exposed to inappropriate financial information and problematic consumption behaviors.

■ Conclusion

This study investigates the financial literacy knowledge, attitudes, and practices of Chinese junior and senior high school students through a standardized questionnaire survey and descriptive statistical analysis, clarifies the current situation and existing prominent problems of adolescent financial literacy in China, and deeply analyzes the main influencing factors from the perspectives of school, family, and society.

The main conclusions of the study are as follows: first, the overall level of financial literacy of Chinese junior and senior high school students is at a medium level. Most students have basic financial awareness (such as recognizing currency and distinguishing income and expenditure) and relatively rational financial attitudes towards money and consumption, but they generally lack systematic and professional financial knowledge and standardized financial management practices. Second, the financial literacy level of junior and senior high school students shows significant differences with the change of grade, family education background, and school financial education resources, as the proportional differences in these variables in the sample are presented in Table 1: the higher the grade, the better the financial literacy performance; adolescents from families with regular financial communication have more rational financial attitudes and behaviors; students in schools with financial education resources have a higher mastery of basic financial knowledge. Third, school financial education, family financial guidance, and social financial environment are the three main factors affecting the financial literacy of junior and senior high school students, and the three factors interact and jointly restrict the improvement of adolescent financial literacy level in China.

This study also has several limitations that need to be acknowledged. First, the sample size is relatively small, and the sampling is restricted to the Beijing Academy, which may limit the generalizability of the research findings to the broader Chinese adolescent population. Future research could expand the sampling scope to multiple regions across China (e.g., eastern, central, and western regions) and multiple schools of different types to improve the representativeness of the sample. Second, the research method is limited to quantitative research (questionnaire survey), and qualitative research methods, such as in-depth interviews and case studies, are not used, which prevents a more in-depth understanding of the psychological activities and specific reasons behind adolescents' financial behaviors. Third, the research only focuses on the cross-sectional current situation of adolescent financial literacy and does not conduct long-term tracking research on the dynamic changes

of adolescent financial literacy with age and educational experience.

Based on the research conclusions and the existing problems of Chinese adolescent financial literacy, targeted improvement suggestions are put forward from three aspects: school, family, and society. From the school's perspective, it is necessary to set up specialized financial education courses, compile grade-targeted teaching materials, strengthen teacher professional training, and carry out diversified financial education practical activities. From the family perspective, parents should set a good example of rational consumption, strengthen financial communication with their children, and intentionally cultivate their children's independent financial management ability through pocket money management and budgeting practice. From the social perspective, it is necessary to strengthen the publicity and popularization of adolescent financial knowledge, improve the social financial education system, and strengthen the supervision of the consumer market and financial information related to adolescents, to create a good social environment for the improvement of adolescent financial literacy.

The improvement of Chinese adolescents' financial literacy is a systematic project that requires the joint efforts and close cooperation of schools, families, and society. Only by forming a joint force of financial education can we effectively help adolescents establish correct financial concepts, master systematic financial knowledge, and cultivate standardized financial behaviors, laying a solid foundation for their rational financial decision-making and long-term economic well-being in adulthood.

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The Role of Liquid Biopsy in Early Detection and Management of Pancreatic Cancer

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ABSTRACT: Pancreatic cancer (PC) has a poor prognosis largely due to detection at a late stage, which limits effective treatment options. This secondary analysis reviews liquid biopsy in the early detection and management of PC, since improving survival outcomes requires earlier detection. Liquid biopsy may identify small tumors early and enable analysis of circulating tumor DNA (ctDNA), circulating tumor cells (CTCs), and other biomarkers. The noninvasive nature allows repeated sampling and continuous surveillance. A thorough review of research articles and clinical studies reveals that liquid biopsy may help determine treatment options, recurrence rates, survival outcomes, tumor monitoring, and early detection. Levels of ctDNA, KRAS, PLF, and CTC-AXL and their presence (+/-) indicate prognosis and survival outcome, with positive levels showing poor prognosis. Its noninvasive nature also guides treatment strategies, and ctDNA may determine recurrence pre- or post-surgery. Although studies are still being conducted, liquid biopsy shows promise as a supplementary tool for early detection and tumor monitoring and could be combined with AI or imaging to improve detection rates and patient outcomes.

KEYWORDS: Biomedical and Health Sciences, Immunology, Pancreatic Cancer, Early Detection, Liquid Biopsy.

■ Introduction

Pancreatic cancer occurs in the pancreas, a gland approximately 6 inches in length, between the stomach and spine, which regulates digestion through the secretion of enzymes and regulates blood glucose via insulin and glucagon production. Pancreatic Ductal Adenocarcinoma (PDAC) is the most common PC, with over 90% of patients showing a mutation in the KRAS oncogene. PDAC is one of the deadliest cancers and is estimated to become the second-leading cause of cancer-related death by 2030.¹ Due to an atypical early stage, PDAC is often detected very late, when limited treatment options lead to a 5-year survival rate of 13%.²

Effective treatment options and early detection methods are essential for improving PC prognosis and survival rates. Early symptoms like fatigue and weight loss are often ignored.³ If the tumor is limited to the pancreatic ducts and is around Stage I, 5-year survival rates may reach up to 80.4%, in some cases.⁴ Despite advances in detection and treatment methods, 10% - 20% of patients are diagnosed in the metastatic stage when the cancer has spread throughout organs and vasculature, leading to poor survival outcomes.³ Thus, an improvement in early detection may lead to better survival rates since more treatment options could be available.

Liquid biopsy is a noninvasive test that analyzes circulating tumor cells (CTCs) and tumor-derived material, such as circulating tumor DNA (ctDNA) in body fluids like blood, stool, saliva, and urine. It provides a convenient and repeatable means for continuous monitoring. It could be applied in early diagnosis and management of PC, including personalizing treatment, monitoring tumor growth and recurrence, and estimating survival rates.⁵ Liquid biopsy may detect tumor-derived biomarkers that could indicate early disease, even when tumors are not yet visible on imaging.⁴

This review highlights liquid biopsy as an emerging tool for early detection and management of PC. It focuses on how liquid biopsies may ameliorate early detection and treatment methods and explores the benefits and challenges of liquid biopsies. PC is one of the deadliest cancers, not solely due to the lack of treatment methods, but largely due to late detection associated with its asymptomatic early stage. Standard imaging techniques have trouble detecting PC during its early stage, which leads to limited treatment options in the future when it is too late. Liquid biopsy may offer a promising approach for improving early detection and opening up more treatment options.

■ Methods

The purpose of this review is to examine how liquid biopsy may improve early detection, tumor monitoring, treatment modalities, and survival outcomes for PC patients. This review article is a qualitative secondary analysis of published data and completed or ongoing trials, consisting of 36 sources total. Journal articles were systematically searched using PubMed, Google Scholar, and Litmaps, while clinical studies were identified using ClinicalTrials.gov. Ethnic and demographic data were obtained using the NIH website. Studies from 2020 to 2025 were included to ensure the inclusion of recent research. The following keywords were used to locate relevant articles: Pancreatic Cancer, Liquid Biopsy, ctDNA, CTC cells, Early Detection, Immunology, Biomedical and Health Sciences, and Pancreatic Cancer Treatment. Inclusion criteria consisted of studies related to PC that involved liquid biopsy for early detection, monitoring, or treatment applications. Only studies published in English were included. Exclusion criteria consisted of studies without the specified keywords, non-peer-reviewed sources such as blogs and opinion pieces, and studies related

to cancers other than PC. This review focuses on the scope of liquid biopsy rather than providing a detailed discussion of standard treatment strategies. Instead, it shows the potential of liquid biopsies in the early detection and management of pancreatic cancer. As this review focused on the advancement and scope of liquid biopsy in clinical application, there may be potential bias toward highlighting the positives of liquid biopsy over its limitations. Because this study is a literature review and did not involve human subjects, formal ethical approval was not required. Flowcharts, diagrams, or illustrations were created using BioRender.

PC Types, Causes (genetic association), Current Treatment Strategies, and Clinical Outcome:

Types of PC:

The two types of PC are endocrine tumors (also known as neuroendocrine tumors) and exocrine tumors. These types are formed based on the tumor origin. Endocrine tumors begin in the endocrine cells located in the pancreas. On the other hand, exocrine tumors arise from the ducts and glands of the pancreas. Pancreatic Ductal Adenocarcinoma (PDAC) is an exocrine tumor and one of the most common PCs.⁶ 90% of patients express the KRAS oncogene mutation (Figure 1).¹

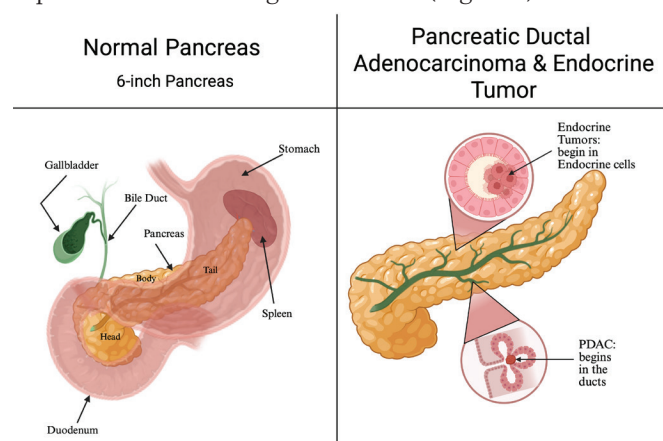


Figure 1: Normal versus cancerous pancreas. This illustration compares a healthy pancreas with one affected by PDAC and endocrine tumors, showing tumor growth, tumor location, and duct obstruction (Created using BioRender).

PDAC is the most aggressive and fatal PC due to its asymptomatic early stage, late detection, thick stroma, and the utilization of autophagy. Detection in the metastatic stage limits treatment options. The thick stroma surrounding the tumor acts as a shield and prevents any drug or medication from entering the tumor microenvironment (TME). Activating mutations in KRAS, along with alterations in TP53, SMAD4, and CDKN2A, allow the tumor to grow rapidly without control. With the help of autophagy, cancer cells have a self-repair and recycling system that allows them to survive with low nutrients and oxygen.⁷

Epidemiology and Risk Factors:

The average diagnosis age for PC is 65 years in the United States, but cases are rising in young women. PC is mainly diagnosed in African American individuals (15.9%), followed

by White (13.4%), Hispanic (12.2%), and Asian individuals (10.3%).⁸

In most cases, the cancer occurs at the head of the pancreas rather than the body or tail. The most common symptoms include decreased appetite, indigestion, pale stool, dark urine, jaundice, fatigue, back pain, and abdominal pain. However, these are general symptoms that are often taken for granted, leading to late detection and poor survival rates due to limited treatment options. PC can occur due to smoking, heavy drinking, chronic pancreatitis, obesity, diabetes, and poor diet choices. Family history, germline mutations (*BRCA 1/2*, *PALB2*), and syndromes like Hereditary Breast and Ovarian Cancer Syndrome (HBOC), Lynch Syndrome 2, and Peutz-Jeghers can also lead to PC.⁹

Genetic Association:

The following gene mutations are linked with pancreatic cancer: *KRAS*, *CDKN2A*, *TP53*, *SMAD4*, *BRCA1/2*, *STK11*, *PRSS1*, Lynch Syndrome, and *ATM*. The *KRAS* oncogene is found in about 90% cases as the most common mutation, and it aids in pancreatic cancer progression. *STK11* (Peutz-Jeghers syndrome) and *PRSS1* pose a high lifetime risk for pancreatic cancer patients. *ATM* and *BRCA1/2* are found in familial pancreatic syndromes and pose a moderate risk. *TP53* and *SMAD4* are mutated in most cases. *TP53* and *CDKN2A* interrupt the DNA's healing process, while *SMAD4* is an indicator of poor prognosis. Lynch Syndrome genes increase the risk of pancreatic cancer.¹⁰ These gene mutations help initiate tumor progression and increase the risk of pancreatic cancer, so genetic testing is recommended for patients, especially for those who have a family history.¹¹

Current Treatment Strategies:

Treatment options for PC include surgery, radiation therapy, chemotherapy, and immunotherapy. However, surgery is only possible for cancer that hasn't spread throughout the body. When considering surgery, the stage and cancer recurrence are reviewed. The four main stages of PC are resectable, borderline resectable, locally advanced, and metastatic. In resectable PCs, the tumor is fairly localized, making surgery possible. In borderline resectable and locally advanced PC, the tumor can be removed, but cancer cells that have spread remain. Surgery isn't possible in metastatic PCs due to widespread cancer. Besides surgery, treatment, and therapies can improve the quality of life.¹²

Surgery: The three main PC surgeries include the Whipple Procedure, Total Pancreatectomy, and Distal Pancreatectomy.¹² The Whipple Procedure removes the head of the pancreas, gallbladder, part of the bile duct, and duodenum. Distal Pancreatectomy removes the body and tail of the pancreas.¹³ Total Pancreatectomy removes the entire pancreas.¹² A study at the Pakistan Kidney and Liver Institute (2018–2019) showed higher survival rates of the Whipple Procedure than the Distal Pancreatectomy, but with more complications such as delayed gastric emptying and pancreatic fistula.¹³

Radiation Therapy: Radiation therapy kills cancer cells through high-energy X-rays.¹² It can prevent the tumor from

growing by causing DNA damage and can be targeted to the affected area (local control). However, the benefits of radiotherapy alone are uncertain, and it does not improve overall survival. The old methods include 2D-radiation and the box technique. They work with large areas using low doses (40–60 Gy), lack precision, and have many side effects. A newer form of radiotherapy is Stereotactic Body Radiotherapy (SBRT). SBRT uses imaging for precision, high biologically effective doses (BED > 70 Gy), smaller fields, has survival outcomes of 14–20 months with little to no side effects, and provides local control. A study shows a 1-year survival rate of 96.4% and a 2-year survival rate of 70.8%. More trials are needed to confirm its effectiveness. 5-FU + Radiotherapy or Gemcitabine + Radiotherapy have an overall survival rate of 8–15 months.¹⁴

Chemotherapy: Chemotherapy prevents tumor growth using drugs that target various mechanisms/pathways of cell division and metabolism.¹² It kills cancer cells, prevents recurrence and tumor growth, and downstages the tumor for surgery. The most common types of chemotherapies are FOLFIRINOX, Gemcitabine, Gemcitabine + Nab-Paclitaxel, modified FOLFIRINOX, Gemcitabine + Capecitabine (GemCap), Nanoliposomal Irinotecan + 5-FU, and Olaparib. FOLFIRINOX is the strongest chemotherapy regimen that uses a combination of drugs (5-Fluorouracil, Folinic acid, Irinotecan, and Oxaliplatin) and has a survival rate of 11 months. Gemcitabine is weaker, with Gemcitabine + Nab-Paclitaxel providing a survival rate of 8.5 months. To prevent recurrence post-surgery, modified FOLFIRINOX is used, showing a 54-month survival rate. Chemotherapy helps slow disease progression, extend survival time, and improve quality of life, but it doesn't cure cancer.¹⁵

Immunotherapy: Immunotherapy helps the immune system fight cancer cells by activating T cells. In PDAC, the thick desmoplastic stroma, consisting of matrix collagen and fibronectin, does not allow immune cells to access the tumor microenvironment. PDAC is one of the immunologically 'coldest' tumors with high *PD-L1* expression and low MHC-I expression. This means that the tumor is highly resistant to immune-based therapies. With chemoradiation therapy or radiotherapy, PDAC can become a 'hot' tumor with high MHC-1 and low *PD-L1* expression. This allows T cells to recognize the cancer better and attack it. Thus, immunotherapy doesn't work on its own for PC, but combining it with chemoradiation or radiotherapy provides a better response.¹⁶

Current Treatment Outcomes:

After surgery, the median five-year survival rate increases to 20%–30%; however, there are high recurrence chances of 70%–80%, so neoadjuvant chemotherapy is given for borderline resectable and locally advanced patients before surgery (Table 1).¹⁵

Table 1: Comparison of pancreatic cancer treatments and their outcomes. Surgery provides the highest survival rate for resectable tumors, but the cancer has a high chance of recurring even after surgery. Neoadjuvant therapy can downstage borderline tumors, allowing for surgery, while chemotherapy, radiation therapy, and immunotherapy support maintenance of PC (created with data from^{14–17}).

Treatment	Used For	Effectiveness	Notes
Surgery	Resectable pancreatic cancer	Five-year survival rate increases to 20%–30%	70%–80% recurrence even after surgery
Chemotherapy or Neoadjuvant therapy	Metastatic, Borderline, or Locally Advanced Pancreatic Cancer Controversial for Resectable pancreatic cancer	Downstage the tumor by 60%, making it applicable for surgery	Often given before surgery to reduce the tumor
Radiation therapy	Locally Advanced or Borderline Cancer	High-energy X-rays kill cancer cells	Average overall survival: 8.5 months
Immunotherapy	Recurring Cancer Borderline, Locally Advanced, or Metastatic pancreatic cancer	Fight the tumor using the T cell defense mechanism	Often combined with Chemoradiation or chemotherapy due to a weak immune response to pancreatic cancer

Other treatment modalities can include chemoradiation therapy and targeted therapy.¹² Targeted therapy attacks genes like *KRAS*, *BRCA1/2*, the *RET* gene, the *NTRX* gene, and the *BRAF V600E* gene. This form of therapy doesn't benefit alone but is often combined with other therapies. Using targeted therapy, specific genes are targeted, which could help reduce tumor microenvironment barriers.¹⁸ Neoadjuvant therapy can downstage tumors by 60%, making surgery possible.¹⁷

However, it is important to consider that in most cases, combination treatment is used instead of using one of the treatment methods alone. As shown in the data from Table 1, the main treatment modalities have a low survival rate when used alone, so combining treatments improves results and overall survival. Chemotherapy, chemoradiation therapy, and radiotherapy are other treatments combined with surgery or immunotherapy. Although these treatment strategies provide various survival benefits, their effectiveness is strongly dependent on early diagnosis. Many of these treatments, especially surgery, are only possible during the early stages. When PC is detected late in the metastatic stage, treatment options become limited, leading to poor prognosis and survival rates. Thus, improving early detection is critical, as it may directly increase treatment options and overall treatment effectiveness.¹⁹

Standard Detection Methods:

MRI, EUS, CT scan:

Traditional imaging includes Magnetic Resonance Imaging (MRI), Endoscopic Ultrasonography (EUS), and Computed Tomography Scan (CT).² MRI scans can detect changes in the ducts often before they exist, without any harmful radiation, and they can differentiate between various soft tissues. However, they often cannot detect small, microscopic tumors. They take a long time to complete; thus, during the process, a person's breath can blur the picture or distort the image when the pancreas moves.²⁰ A study (2007–2020) from Onomichi General Hospital found that EUS detected 45.5% of stage 0 and 81.8% of stage I tumors, outperforming CT and MRI.

EUS has a 94.4% sensitivity and can identify Main Pancreatic Duct (MPD) abnormalities; however, its accuracy depends on the operator's skill and often requires a biopsy afterwards.²¹ CT is fast, available, and has good imaging due to its proximity. However, it uses radiation and can miss small tumors.²² In conclusion, not all traditional imaging modalities use ionizing radiation, and in most cases, these imaging methods are unable to detect tumors or aren't sufficient for detection by themselves. Figure 2 shows that liquid biopsy is often able to detect the small tumors missed by the other imaging modalities.

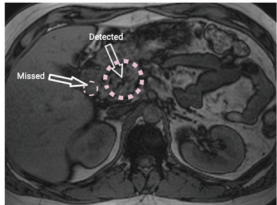
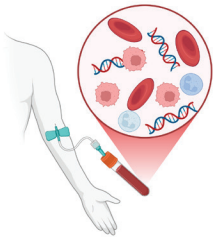
MRI, CT Scan, EUS	Liquid Biopsy
	
Traditional imaging methods can detect larger tumors, but tend to miss small lesions.	Liquid Biopsy can detect biomarkers like ctDNA and CTCs, or mutations in the blood.

Figure 2: Schematic representation of diagnostic tools for pancreatic cancer. This illustration compares traditional detection methods with liquid biopsy. While traditional imaging methods like MRI, CT scan, and EUS detect larger tumors, liquid biopsy uses biomarkers like ctDNA, CTCs, and *KRAS* mutations to detect small lesions (Created using BioRender).

Finding cystic lesions like Intraductal Papillary Mucinous Neoplasm (IPMN), Mucinous Cystic Neoplasm (MCN), and Pancreatic Intraepithelial Neoplasia (PanIN) is another early detection method.¹⁹ IPMN and MCN are easy to detect through imaging, unlike PanINs, which require surgery. These cysts are often discovered unintentionally, and they can develop into pancreatic cancer. However, many of the cysts don't turn into cancer and are harmless. Having different gene mutations like *KRAS* and *GNAS* for IPMN and MCN means that these lesions need to be examined during endoscopy. Many people get surgery for these cysts, which is often (in 78% cases) unnecessary.²³

Biomarkers:

Biomarkers are proteins or DNA/RNA that indicate signs of a tumor at an early stage and show tumor progression. They can be used to detect cancer, determine the type of cancer, and monitor cancer prognosis. Circulating free DNA (cfDNA) is present in the bloodstream and is released from natural cellular processes such as apoptosis and necrosis. cfDNA originates from all cells in the body. However, in cancer patients, the tumor-derived fragments of cfDNA are known as ctDNA. These ctDNA fragments contain mutations like *KRAS*, *TP53*, and *SMAD4*, and the presence of these mutations distinguishes the ctDNA from the regular cfDNA by indicating that this is a tumor-derived fragment.²⁶ Common PC biomarkers include CTCs, ctDNA, *TP53*, *SMAD4*, *BRCA 1/2*, *CDKN2A*, *CA-19-9*, and *KRAS*, which may provide insights into the detection

of PCs.²⁴ Liquid biopsy uses biomarkers to detect PCs. It uses body fluids to analyze ctDNA, CTCs, and other biomarkers. CTCs are actual cancer cells that float in the bloodstream, while ctDNA are bits of DNA that originate from the tumor. ctDNA and CTC cell analysis may determine detection, the cancer stage, recurrence, and survival outcomes.²⁵ Identifying biomarkers like Carbohydrate antigen 19-9 (CA19-9) and *KRAS* aids pancreatic cancer detection and monitors treatment. However, CA19-9 isn't native to PC, so it may be increased in patients who smoke or have inflammation.²⁶ The *KRAS* oncogene can be seen in many patients during the early stage of PC, aiding in early detection. Yet, there are low levels of the *KRAS* gene in the blood and cystic juice, most of which aren't specific to PC.⁸ According to a recent study done on biomarkers, biomarkers like *MXRA8*, *LTBP2*, *PLA2G1B*, and *KIRREL2* have shown a specificity of 84% and a sensitivity of 95%, which is significantly better than CA 19-9 for PC detection.²⁴ Overall, these biomarkers could indicate the formation of PC, and detecting them early through liquid biopsy may improve the early detection of PC. To avoid false positive results, multiple biomarkers must be analyzed.

Novel Detection Methods:

Liquid Biopsies:

A non-invasive test, liquid biopsy utilizes body fluids to detect the Circulating Tumor Cells (CTCs) and Circulating Tumor DNA (ctDNA) fragments in the blood. Both provide a molecular analysis of the prognosis. They could be used for early detection, staging, recurrence, monitoring, and personalizing treatment.²⁵ Liquid biopsy aids in early detection because ctDNA may provide information on mutations or specific characteristics of the patient's tumor. ctDNA-based biopsies help differentiate between pancreatitis patients with the *KRAS* gene and between PDAC patients with the *KRAS* gene. However, there is a limited amount of ctDNA in the Circulating Free DNA (cfDNA), so ctDNA is often hard to detect.²⁶ An abundance of CTCs portrays an advanced stage of PC, which determines if chemotherapy should be given before surgery. Moreover, CTCs help provide information about disease stage and recurrence possibilities.²⁷ Staging is vital in determining treatment options for PC patients. CT scans and MRIs often can't detect molecular and microscopic details of the tumor, so a liquid biopsy is preferred for monitoring the tumor and its progression. Depending on the gene mutations present, treatment and the type of chemotherapy could be suggested to personalize treatment for the patient.² The noninvasive nature of liquid biopsy allows for continuous monitoring and for the test to be repeated easily through any bodily fluid (blood preferred). As shown in Figure 3, liquid biopsy detects ctDNA and CTCs using clinically applied bodily fluids like blood, stool, saliva, and urine to allow for early diagnosis, treatment monitoring, and improve survival rates.⁵

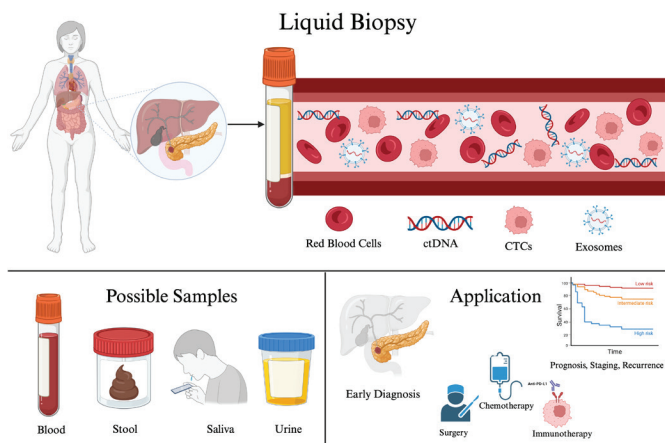


Figure 3: Liquid biopsy in pancreatic cancer. This illustration demonstrates liquid biopsy biomarkers, including ctDNA, CTCs, and exosomes, the bodily fluids that can be used during the biopsy, and the main clinical applications (Created using BioRender).

Breath Analysis:

Breath analysis for the diagnosis of pancreatic cancer and many other cancers is a fairly new concept. In the human breath are volatile organic compounds (VOCs) that are created by the body's metabolism. When diagnosed with PDAC or any cancer, the VOCs ratio changes. Acetone dimer and Dimethyl sulfide are two main VOCs associated with pancreatic cancer that increase if a tumor is present. Studies show that Acetone dimer was able to detect pancreatic cancer better than CA19-9 (0.91 vs. 0.796), and when Acetone dimer was combined with CA19-9 in detection, 96.7% sensitivity was shown. Breath analysis may open doors to easier non-invasive detection methods that have an increasing accuracy; however, research still needs to be done to provide assurance.²⁸

Clinical Studies on Liquid Biopsy in Pancreatic Cancer:

The potential of liquid biopsy has been subject to several clinical trials, particularly analyzing Circulating Tumor DNA (ctDNA). One such study is GUIDEMRD (*Guiding Multi-Modal Therapies Against Minimal Residual Disease By Liquid Biopsies*) (NCT06102889), a clinical trial in Sweden that examines the relationship between positive ctDNA after surgery or chemotherapy and recurrence of PDAC via a liquid biopsy. Patients are required to provide a blood sample and must be scheduled for surgery. To prevent skewed results, the Electronic Health Record and the National Health Record are also checked. The aim is to test the role of post-surgery ctDNA in predicting survival rates, recurrence chances, and treatment options.²⁹

In Germany, the *Prognostic Role of Circulating Tumor DNA in Resectable Pancreatic Cancer, or PROJECTION trial* (NCT04246203), investigates ctDNA to predict DFS and recurrence of RPC cancer post-surgery. Patients with RPC are monitored for 36 months or until death or cancer recurrence. A liquid biopsy will be done before surgery and 14 days post-surgery to monitor for ctDNA. The patients will be divided into two groups: Group A (with ctDNA present) and Group B (without ctDNA present). This study focuses on dis-

covering the DFS for patients with RPC through the presence of ctDNA.³⁰

Similarly, *Liquid Biopsy for ctDNA in Peritoneal Lavage and Blood in Pancreatic Cancer (LIPAC)* (NCT05400681) examines survival rates and the presence of *KRAS* mutations in peritoneal lavage fluid (PLF) or ctDNA. Having a *KRAS* mutation with positive PLF or positive ctDNA predicts poor prognosis and worse survival. Blood samples are collected before and after surgery to identify mutations and their relation to survival outcomes. Enrolled subjects must be at least 18 years old and PDAC patients with a pancreatic resection specimen. Therefore, the purpose is to portray the scope of liquid biopsy and check PLF and ctDNA for mutations to estimate survival rates.³¹

Pancreatic Cancer Initial Detection Via Liquid Biopsy (PANCAID) (NCT06283576), a study in Sweden, gathers results of the PANCAID blood test in early detection of PC, predicting recurrence, and tumor staging. IPMN lesions suggest the presence of cancer or the possibility of PC development. Enrolled subjects must be at least 18 years old and have a chance of increased IPMN levels. This blood test aids in the early detection of PC and recurrence rates, providing patients with the best possible treatment.³²

Additionally, *Mutation of K-RAS, CDKN2A, SMAD4, and TP53 in Pancreatic Cancer: Role of Liquid Biopsy in Pre-operative Diagnosis* (NCT03524677), in Ireland and Rome, analyzes gene mutations (*KRAS*, *CDKN2A*, *SMAD4*, *TP53*, *MET*, *EDIL3*, *BRCA1/2*, *RAB27A*) in blood samples to guide tumor staging and treatment options, and to predict cancer recurrence, prognosis, and surgery outcomes (DFS and OS). Before surgery, a blood sample from a group of 50 participants receiving pancreatectomy for non-metastatic PC will be collected. Patients must be 18 years or older and diagnosed with non-metastatic cancer to participate. Thus, this study focuses on the relationship between gene mutations and cancer results or prognosis.³³

In France, *Liquid Biopsy and Pancreas Cancer: Detection of AXL(+) CTCs (CTC-AXL-PANC) (CTC-AXL-PANC)* (NCT05346536) compares the CellSearch and EPIDROP methods that detect AXL genes in ctDNA via liquid biopsy. AXL is a protein generally released by CTCs that correlates with a poor prognosis. To enroll in the study, patients must be 18 years old and have remote metastases with Pancreatic Cancer.³⁴

South Korea's *Verification of Predictive Biomarkers for Pancreatic Cancer Treatment Using Multicenter Liquid Biopsy* (NCT04241367) investigates the *KRAS* gene in detection and prognosis estimation for PC patients via liquid biopsy. Enrolled patients must be pathologically confirmed with PDAC and must provide a blood sample. The droplet digital PCR (ddPCR) will examine the ctDNA for the *KRAS* gene and biomarkers. The study examines the correlation between the mutated gene and PDAC prognosis, cancer recurrence, response to treatment, or detection.³⁵

Finally, Austria's *Liquid Biopsy (ctDNA) Guided Treatment in Localized Pancreatic Cancer: Neoadjuvant CTX vs. Upfront Surgery (LIQUIPANC)* (NCT06391892) tests the PFS and OS

of LAPC patients after receiving chemotherapy before typical surgery. This novel pathway is chosen based on the liquid biopsy results through ctDNA analysis from a blood sample. Patients must be 18 years or older to participate. Overall, the study examines the effectiveness of their new strategy in improving the prognosis for LAPC patients.³⁶

Collectively, these trials reflect the use of liquid biopsy in predicting quality of life, recurrence chances, staging, metastasis, and guiding treatment. All the trials utilized blood samples, gene mutations, and biomarkers to predict survival rates and aid in early detection and personalizing treatment. The following are common biomarkers and mutations used for detection and recurrence: CTCs, ctDNA, *KRAS*, CA 19-9, *BRCA 1/2*, *TP53*, *SMAD4*, and *CDKN2A*. Despite the diverse stages of pancreatic cancer studied—from resectable to metastatic—and varying sample types, these clinical trials collectively highlight the liquid biopsy's possible clinical potential in enhancing early detection, guiding treatment decisions, and improving prognosis. Results from these clinical studies, once completed, are likely to provide avenues for early detection and monitoring of prognosis to improve survival rates in this deadly disease (Table 2).

Table 2: Summary of clinical trials that employ liquid biopsy in the early detection and management of pancreatic cancer. These trials explore biomarkers like ctDNA, *KRAS*, and CTCs to predict recurrence rates, monitor tumors, early detection, and guide treatment options. Created using data from clinicaltrials.gov.

Clinical Trial ID #	Mutations & Biomarkers	Purpose	Samples
NCT06102889	ctDNA	Detect recurrence after treatment	Blood
NCT04246203	ctDNA	Predict recurrence & quality of life before and after surgery	Peripheral blood
NCT05400681	<i>KRAS</i> ; <i>CDKN2A</i> <i>SMAD4</i> ; <i>TP53</i> PLF+ctDNA	Correlate gene mutations in ctDNA and PLF with survival outcomes	Blood Peritoneal Lavage (PLF)
NCT06283576	<i>BRCA2</i> IPMNs	Use of the PANCAID blood test for recurrence, early detection, and development of PC	Blood
NCT03524677	ctDNA <i>KRAS</i> ; <i>CDKN2A</i> <i>SMAD4</i> ; <i>TP53</i> <i>MET</i> <i>BRCA1/2</i> <i>EDIL3</i> <i>RAB27A</i>	Predict staging, analyze tumors, and guide treatment in non-metastatic PC via liquid biopsy	Blood
NCT05346536	CTCs AXL	CTCs with AXL to predict recurrence, treatment effectiveness, and detection	Blood
NCT04241367	<i>KRAS</i> ctDNA <i>PD-L1</i>	Use <i>KRAS</i> gene biomarkers for detection and prognosis	Blood
NCT06391892	CA 19-9 <i>KRAS</i> ctDNA	Guide chemotherapy and predict survival in localized pancreatic cancer	Blood

■ Discussion

PC remains one of the deadliest cancers due to its late detection in the metastatic stage. Treatment methods like surgery, chemotherapy, radiotherapy, targeted therapy, and immunotherapy are used to manage pancreatic cancer. Neoadjuvant therapy can be used to downstage the tumor to make it accept-

able for surgery. Detection methods like MRI, EUS, and CT scan often miss small lesions that could indicate tumors at an early stage. Liquid biopsy may detect the small lesions that are often missed by standard detection methods. Its noninvasive nature allows for repeated use for tumor monitoring. Liquid biopsy may discover new gene mutations related to pancreatic cancer, and a variety of body fluids could be used. Overall, the use of liquid biopsy may improve the outcome, detection, and maintenance of pancreatic cancer and may potentially surpass imaging. Breath Analysis is another emerging novel method that may take early detection to a new level; however, further research and trials are required before it can be clinically applied.

Despite the various treatment and decision methods, pancreatic cancer survival rates and outcomes have not improved. This is mainly due to the following two reasons: 1) desmoplasia, 2) late detection when tumors are metastatic and unresectable. Current standard imaging detection methods (MRI, EUS, and CT scan) are inadequate to detect tumors at an early stage since they often miss small lesions. Liquid biopsy may detect small lesions and discover new gene mutations linked to pancreatic cancer, which may outperform imaging. It offers a noninvasive way to detect and monitor PC by detecting CTCs, ctDNA, or other biomarkers in blood and other body fluids, and its noninvasive nature avoids potential infections and allows for the test to be repeated multiple times. Since analysis of a few biomarkers could sometimes lead to false-positive or false-negative results, multiple biomarkers are analyzed to increase the accuracy of PC detection.²⁴ Thus, liquid biopsy may improve survival rates through its improved early detection and recurrence methods, and easy monitoring to check PC metastasis.

This review examined recent clinical trials on the application of liquid biopsy in detecting recurrence and monitoring pancreatic cancer. Although all trials were either ongoing or the results were not yet available, definitive conclusions could not be drawn, but some patterns emerged. Most studies showed that ctDNA analysis was used to personalize treatment, detect PC early, and predict recurrence. Blood samples were the most common method used in the majority of the studies. These studies mainly focused on early detection and recurrence. The biomarkers and mutations used included CTCs, ctDNA, *KRAS*, CA 19-9, *BRCA 1/2*, *TP53*, *SMAD4*, and *CDKN2A*. These trials highlight the potential of liquid biopsy in improving prognosis and survival through early detection and identification of recurrence rates.

Many studies examine the role of liquid biopsy in guiding treatment, predicting survival outcomes and recurrence rates, as well as facilitating early detection. Several trials analyze ctDNA through blood samples to help in early detection and estimating recurrence pre- or post-surgery. Analyzing the *KRAS* gene, ctDNA, PLF, and CTC-AXL may predict prognosis. The *GUIDEMRD* study and the *LIPAC* study examine ctDNA correlation with poor survival outcomes. Similarly, the (*CTC-AXL-PANC*) trial analyzes the relationship between positive AXL and survival outcomes. Thus, positive or high levels of biomarkers like ctDNA, PLF, CTC-AXL, and *KRAS* genes may indicate poor survival and prognosis. Multiple trials

have been using liquid biopsy analysis to estimate recurrence and guide treatment.

However, the potential outcomes of all the trials may not be the same. For example, some trials are conducted for different stages or types of pancreatic cancer, which include PDAC, non-metastatic, locally advanced pancreatic cancer (LAPC), and resectable pancreatic cancer (RPC). The variation in type or stages leads to varied results and outcomes. Moreover, there are a variety of biomarkers used. Some trials use multiple biomarkers and gene mutations, while others focus on *KRAS* or ctDNA analysis. The *LIPAC* clinical trial uses PLF and blood as samples to analyze. The rest of the trials only use blood samples to conduct research. Many of the trials use liquid biopsy as an additional factor to monitor treatment or predict recurrence. However, the *LIQUIPANC* study uses ctDNA to determine a treatment path that varies from the traditional path. This study performs chemotherapy before surgery based on ctDNA analysis from a liquid biopsy. Here, ctDNA analysis is used to diagnose and test a different approach to the treatment of PC. Varying study designs, cancer stages, and biomarker choices make it difficult to directly compare results. Moreover, as many trials don't have published results and are still ongoing, the outcomes aren't confirmed, limiting the ability to fully understand the effectiveness of liquid biopsy in clinical situations.

Overall, these studies demonstrate clinical application of liquid biopsy to improve prognosis, treatment strategies, early detection, recurrence prediction, OFS, DFS, and PFS. While some studies were conducted during different stages of PC and analyzed a different scope of biomarkers, some studies discovered a correlation between high ctDNA, PLF, CTC-AXL, and *KRAS* mutations and poor prognosis or survival. Analyzing multiple biomarkers helped guide treatment decisions, such as determining whether chemotherapy should be administered before or after surgery. These findings highlight the growing role of liquid biopsy in improving early detection, treatment planning, and monitoring of pancreatic cancer. However, ctDNA is normally low in early stages, which creates a detection challenge for liquid biopsy. Liquid biopsy is not directly compared to MRI or CT scans in these trials, and it is not widely used in real hospitals, as it is still a novel detection method. Combining liquid biopsy with imaging and AI could potentially improve detection rates and may provide more assurance of not missing the tumor during early stages. A combination approach may create a more cohesive and stronger detection method.

Future studies should explore the field of liquid biopsy in detail. Apart from CTCs and ctDNA, exosomes, extracellular vesicles, and microRNA, other biomarkers may play a role in improving early detection and monitoring of PC. Future trials should connect their study to all stages of pancreatic cancer and see which treatment approach fits best for which stage. Liquid biopsies may be combined with AI analysis in the future, and combination methods could be researched. For example, immunotherapy, chemotherapy, and radiotherapy may be evaluated, and their effects could be monitored through liquid biopsy.

■ Conclusion

This study examined the application of liquid biopsy in the diagnosis, treatment, and management of pancreatic cancer. Liquid biopsy provided significant potential for improved early detection, recurrence prediction, treatment guidance, survival outcomes, and tumor monitoring. ctDNA and CTCs analysis helped identify new biomarkers related to PC, like the *KRAS* gene. A biomarker amount or its sign (+ or -) could correlate with poor survival rates and prognosis. This is supported through the analysis of ongoing trials like *LIPAC*, *GUIDEM-RD*, and (*CTC-AXL-PANC*). The noninvasive nature of liquid biopsy allowed it to be repeated easily when required. However, a significant challenge remains at early stages, as ctDNA and CTCs may be insufficiently abundant for reliable detection, limiting diagnostic sensitivity. In the future, liquid biopsy could be combined with AI to enhance biomarker analysis and to identify new gene mutations. The use of other body samples for liquid biopsy and the use of breath analysis in the detection of PC could be explored further. In conclusion, liquid biopsy may enhance early detection, treatment, and management of pancreatic cancer through the analysis of ctDNA and CTCs.

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Genetics, Hormones, and Muscle Growth: A Machine Learning Review

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ABSTRACT: Rising interest in fitness means that more will follow already established programs, but many do not see the same results. AI serves as a way to close the gap between broad programs and individuals, as it can be used to personalize fitness based on the biology of an individual. Through this meta-analysis of various studies of ML models in skeletal and cardiac hypertrophic contexts, it was found that a Generalized Linear Model (GLM) was the most consistently accurate, predicting muscle growth with an AUC of 0.809, while also excelling in transparency compared to other ML models. In applying this, it can be seen that this model is the most efficient in predicting muscle growth, in which a more complete model could include k-means clustering that will allow for the detection of sub-patterns through grouping. These predictions will take in parameters such as protein biomarkers, phenotypic, hormone levels, and genetic data to classify whether a muscle will/won't experience significant muscle growth. It was also found that around 1575 participants would ideally be needed to have enough data for an accurate model that doesn't overfit as well. Through this proposed model/study, machine learning can be utilized universally to progress fitness in individuals.

KEYWORDS: Machine Learning, Computational Biomodeling, Artificial Intelligence, Skeletal Hypertrophy, Algorithms.

■ Introduction

With the rise of those getting into fitness, it's important to understand goals and what types of movements work best for others. Understanding the type of movements that work better based on their hormones and genetics can help save time and help them progress faster to their goals. For example, the *ACTN3* gene, a genetic polymorphism, determines the muscle-fiber compositions and potential strength of an individual, showing that certain movements may benefit those with certain muscle fiber compositions, showing a greater need for fitness individualization.¹ This demand is urgent as interest in gym/fitness clubs has risen by 8% to 77 million individuals possessing a fitness club membership, with this rate set to progressively increase due to social media shifting gym culture to become more accepting.² In addition, the conduction of similar studies has significantly risen post-pandemic, as well, showing a more modern acknowledgement of fitness in everyday life.³

My project involves analyzing existing ML models and studies that analyze muscle attributes as well as relationships between hormone levels, phenotypic traits, genetics, and hypertrophy (the bulkening of muscle tissue/muscle growth), the study will present an ideal method that takes into account these variables and models and apply them to an efficient ML model that can accurately predict this specific relationship that specifically takes into account both muscle growth and hormone and genotype, and the dependent relationship on hypertrophy stemming from these factors. This approach is endorsed across other studies that attempt to identify a relationship between phenotypic/external traits and their dependence on genetics, as supported in Libberecht & Noble's study recommending ML to determine gene function.⁴ Similar research in tissue-related changes, such as cancer detection, has given similar solutions, such as analyzing CT scan data to detect whether scan images

contain the same visual patterns as scans with ovarian cancer, as well as using algorithms to predict its recurrence.⁵ This shows that in the context of tissue analysis, machine learning models such as Deep Learning can be used to predict certain indicators of certain events (prognostic biomarkers in the case of disease).⁶

To continue, the following sections of this report will address important terminology in both the fields of ML and hypertrophy, analysis of existing machine learning models in similar domains related to hypertrophy and genetics. Sequentially, a comparison between the covered methods of all projects' attributes will result in a proposed model that best depicts and predicts the relationship between muscle growth and genetics.

■ Methodology

This paper aims to conceptualize a model and conditions for a study that would accurately create an ML model that predicts hypertrophy based on these genetic factors.

Given the comparative review structure this paper takes on, multiple studies were checked regarding hypertrophy and the algorithms they used to predict for certain genes that signified/were prominent causes for muscle growth, as well as models from studies that predict the muscle thickness/hypertrophic growth itself in addition to predicting whether an individual has the genes or has a high chance of muscle growth in certain areas. Using sites such as Google Scholar, Jstor, and Research Gate to find article files, and searching keywords such as Hypertrophy, Genetics in Hypertrophy, Skeletal Hypertrophy, AI in Hypertrophy, as well as narrow topics specific to the models used in the study, allowed for a more general article in the context of the study. The result was international journals as well as sources on specific topics written by experts in that topic.

This then let me find journals in the National Journal of Bio-tech Information, Frontiers, and Scientific Reports. By looking at the data tables they present and comparing them by their AUC across multiple studies, I was able to evaluate them by the same metric of accuracy.

The researcher found universal methods of verifying the accuracy of ML models across multiple studies, finding that strategies such as finding the area under the receiver curve (AUC) were well-established across multiple studies. It is imperative to acknowledge that AUC comparisons were used as a means of accuracy relative to the hypertrophy context, as an algorithm was being conducted on, due to differences in validation strategies, class balances, and varying types of datasets used in the training of different ML models. Due to this, AUCs have been used as a directional accuracy indicator to theoretically show a model's likelihood of being effective in the context that all models are trained in equal, controlled conditions, rather than an objective accuracy ranking.

Through this, ML was also seen to enhance many previously existing predictive models of hypertrophy or genes that support it as well. The aim is to propose a possible ML model geared specifically towards predicting hypertrophy based on genotype, lifestyle habits reflected through calories and sleep hours, and hormone levels. The following models found will be discussed on the metrics of accuracy, transparency in their use, sensitivity, level of data cleaning needed, and self-correction present within each model's application to determine which best fits this application.

Since there were no physical tools or data extracted by personal methods by the researcher, there were little to no ethical considerations regarding the methodology, as all information was gathered from online sources and compiled to create a proposed accurate method for the goal described.

Model Comparison:
Hypertrophic Cardiomyopathy and Genotype Prediction:

Liang *et al.*'s study used combined learning tree-based models with the existing Mayo and Toronto prediction models to predict sarcomeric genotype positivity. As shown in Figure 1, the study used a random forest (RF), which functions by using multiple decision trees when training the model, in which each tree is built on a random subset of the data/muscle tissue used (bootstrapping) and a random subset of the features tested for. Acting in a flowchart-like structure, its internal nodes represent tests on a feature, in which each branch shows the outcome of the test, where the following leaf nodes represent a classification label, such as the patient being positive/negative. The final prediction is decided through aggregating the predictions of all its individual trees via a majority decision for classification. Through this, the variance and overfitting of a model are reduced.⁷

A Gradient Boosted Decision Tree (GBDT), on the other hand, builds trees one at a time, where every new tree made corrects the errors of the previously made trees.⁸ It focuses on the residuals/differences between the current and predicted values, combining several weak decision trees into a refined learning model. Through this, accuracy typically increases through iter-

atively minimizing a loss function through gradient descent.⁸ To compare with Table 1, an Adaptive Boosting Decision Tree is another boosting decision tree type that adjusts the weights of wrongly classified records, after a set number of iterations/epochs, creating a specific focus on more obscure/complex cases.¹⁰ The different ways these tree-based methods function have also provided different predictive results for the genotype prediction of hypertrophic cardiomyopathy. Figure 1 illustrates the workflow of the tree-based methods used, indicating the formation of the decision tree in the case of the Random Forest method and the step-by-step formation of the decision tree in the case of the boosting method. Table 1 provides a comparative study of the functioning of these tree-based methods, indicating that the Random Forest method provided the best results in terms of accuracy, with an AUC of 0.92 for the prediction of the hypertrophic cardiomyopathy genotype, using the nonlinear multi-variable function of the genetic traits of the subject. This method is best suited to determine the different aspects of the lifestyle that influence the muscle growth potential of the subject. Gradient Boosted Decision Trees perform best in self-correction, although the chances of overfitting are present. Adaptive Boosted trees perform best when the data is clean and normalized.

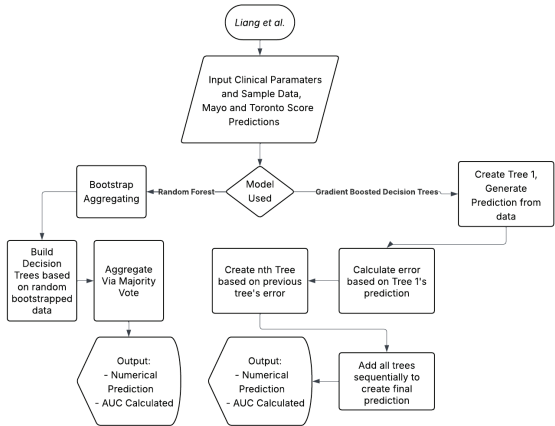


Figure 1: Process for Tree-Based Models in hypertrophic cardiomyopathy prediction: Study on machine learning models in predicting hypertrophic cardiomyopathy risk using Random Forests and Gradient Boosted Decision Trees.²² Created using Lucid

Table 1: The Random Forest achieved the highest accuracy (AUC: 0.92) when predicting for the hypertrophic cardiomyopathy genotype, while Gradient Boosted models excelled in self-correction while having a greater risk of overfitting.²²

Model	Function	Accuracy (AUC)	Strengths	Limitations
Random Forest	Creates multiple decorrelated decision trees on random subsets of provided data and features, aggregated by voted majority.	0.92	• High Accuracy • Robust to overfitting • Able to process complex interactions	• Individual predictions of each decision tree aren't as easily interpretable
Gradient Boosted Decision Tree	Creates sequential decision trees, with each tree focused on correcting the previous trees' errors.	0.87	• High Net Reclassification Improvement, as it is able to correct itself more efficiently. • Able to process complex patterns	• More prone to overfitting • Computationally intensive to train each tree sequentially
Adaptive Boosted Decision Tree	Changes the weights of misclassified instances after each epoch, resulting in future iterations focusing on more difficult problems.	0.88	• Effective and accurate for classes with difficult classification	• Unorganized/noisy data could hamper its accuracy, so data needs to be clean/normalized

In terms of study limitations, the sample size was limited, which may have reduced the potential for noise in the data, potentially hindering its general accuracy if applied at a larger scale (Table 1). The baseline data were collected from 104 patients selected from tertiary referral centers, resulting in a smaller data pool and greater bias. This bias stems from the overall younger age of the patients in the groups tested, resulting in increased positive genotype detection across all models.

In all, this source proves itself valuable to applying ML in hypertrophy in relation to genetics through showing random forests to be the most accurate in relation to established tests regarding the precedence of certain types of genotypes within an individual (Figure 1, Table 1). These findings can then be generally applied to genotypes that are explicitly known for increasing muscle growth. While this could likely be applied similarly, the study itself doesn't address events/parameters known to result in changes in muscle tissue, such as exercise and age, in which the types of cells analyzed were exclusively pathogenic sarcomere mutations.

Gene Biomarker Identification Using Machine Learning:

You and Dong used the ML models of Least Absolute Shrinkage and Selection Operator (LASSO) Regression and Support Vector Machine-Recursive Feature Elimination (SVM-RFE). As outlined in Figure 2, an SVM is first trained on a dataset, in which it works by finding an optimal hyperplane in a high-dimensional space that classifies multiple classes with the widest margin. Data points closest to the hyperplane boundary have their distances measured through support vectors, in which RFE ranks all features based on their importance to the SVM model, which was seen through the ordered common genes of *RASD1*, *CDC42EP4*, *MYH6*, *FCN3*, being seen as strong biomarkers. This happens through using the square of the model's coefficients as weights for each attribute, then removing the least important features, in which the SVM is retrained with these remaining features. This allows the model to find the most discriminative subset of genes.¹¹

LASSO, on the other hand, is a linear modeling technique that does both variable selection and regularization to improve its accuracy and transparency when being interpreted. It adds a penalty equal to the absolute value of the magnitude of its coefficients via L1 Regularization onto typical linear regression models. This categorizes coefficients' importance, as coefficients seen as less important see their values increase/decrease towards zero, in which they are excluded from the model if they do equal zero. When compared to SVM in Table 2, it prioritizes significant predictive factors in a method that also values interpretability, making it ideal for identifying gene biomarkers when classifying whether an individual did/didn't have hypertrophic cardiomyopathy.¹² Figure 2 shows the performance of SVM-RFE and LASSO in gene selection. The performance of the SVM-RFE method is based on the training of the classifier and the iterative removal of the least important features using the magnitude of the weights. The performance of the LASSO method is based on the implementation of the L1 regularization method. As shown in Table 2, the integration of the two methods resulted in the

identification of high-impact genes such as *MYH6*, *RASD1*, *CDC42EP4*, and *FCN3* with an accuracy level of 0.993. The advantage of using the LASSO method is the interpretability of the results, unlike the SVM-RFE method, which requires the training of the classifier.

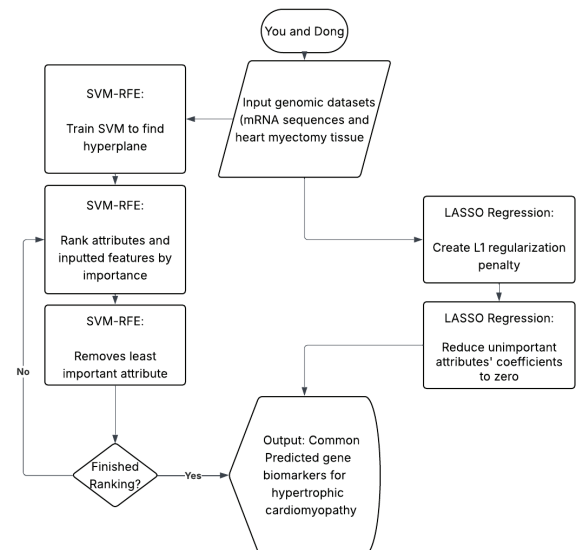


Figure 2: Process for LASSO and SVM-RFE in hypertrophic cardiomyopathy prediction, using machine learning models in predicting gene positivity for hypertrophic cardiomyopathy.⁶ Created using Lucid.

Table 2: SVM and LASSO Metrics: Functions, Accuracy, Strengths, and Limitations. SVM-RFE and LASSO models identified key gene biomarkers with high accuracies and AUCs up to 0.993. LASSO provides greater interpretability through feature selection.⁶

Model	Function	Accuracy (AUC)	Strengths	Limitations
Support Vector Machine - Recursive Feature Elimination	Ranks attributes/inputs by importance, removing the least important features through every iteration for optimization.	When ranking features, commonly predicted genes between both models had AUCs of: 0.954, 0.993, 0.922, 0.710 Using a logistic regression.	Identifies and ranks the most discriminative biomarkers from high-dimensional data.	- Requires a separate classifier. - Not viable on its own.
LASSO Regression	Applies an L1 penalty to make the coefficients of less important attributes zero, creating an optimal model to show which attributes primarily contribute to a certain result.	When ranking features, commonly predicted genes between both models had AUCs of: 0.954, 0.993, 0.922, 0.710 Using a logistic regression.	- Highly interpretable. - Allows for more accurate feature selection	Assumes a linear relationship between attributes and outputs

14 genes were predicted to be biomarkers as a result. In all, only four common 4 genes (*RASD1*, *CDC42EP4*, *MYH6*, *FCN3*) in both models were seen as strong biomarkers (Figure 2, Table 2). After the probabilities of each sample are calculated, the true positive rate (TPR) and false positive rate (FPR) are plotted at different probabilities, creating the ROC Curve using logistic regression, in which the area below it is the AUC. For the 2019 dataset, the AUCs for these 4 common genes were 0.954, 0.993, 0.922, and 0.710 (Table 2). Biases are present within the setup of the study, as one of the datasets was from heart myectomy samples, implying the HCM cases in the training data were severe.

Like the previous study, factors such as age and gender weren't taken into consideration when training the model as

well. From this, specific genes such as *MYH6* were seen to reduce hypertrophy when applied therapeutically, and *FCN3* was seen as a possible use in anti-inflammatory treatments as well.

Deep Learning for Genotype Prediction from Imaging:

Deep Convolutional Neural Networks (DCNNs) were used in Morita *et al.*'s study, using a deep learning structure for processing images, which can be represented as grid-like data, in which Figure 3 outlines this classification process. This is seen as DCNNs automate feature extraction as their initial layers contain convolutional filters to detect features such as edges and specific textures. The following layers take these low-level features to detect specific complex patterns, such as myocardial textures, in this context. Pooling layers reduce the complexity, or dimensionality, of the mapped/visualized versions of these features, making feature detection change less often during shifts. All connected layers at the end of the network combine all extracted features to classify an entity, in this case, checking whether it's positive or negative, as seen in the performance metrics of this model in Table 3. This was used in combination with clinical scores developed using logistic regression and analyzed to see which features significantly affected the model using Grad-CAM. Logistic regression functions through modeling the probability of a binary output based on multiple predictor values. DCNNs were also combined with Mayo and Toronto scales, as well as using variables such as age, echocardiographic measurements, and family history.¹³

Figure 3 shows the DCNN model used in the classification of the genotype features. The first layers are responsible for the identification of the low-level features, such as edges, and the subsequent layers are responsible for the identification of the high-level features, such as myocardial texture. The performance metrics are shown in Table 3, indicating that the DCNN method had an AUC value of 0.86 when the Mayo scores were integrated and an AUC value of 0.84 when the Toronto scores were integrated. The results from the Grad-CAM analysis showed the important features, although the lack of precision may result in biases.

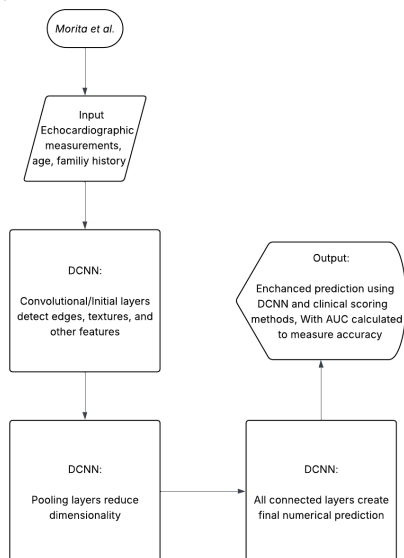


Figure 3: Process for Deep Convolutional Neural Networks in hypertrophic cardiomyopathy prediction.²¹ Created using Lucid.

Table 3: Deep Learning Model Performance Characteristics: DCNN Performance Metrics with Clinical Scales. Deep Convolutional Neural Networks (DCNNs) were able to process echocardiographic images relatively accurately for genotype prediction (AUC: 0.86) but were limited by data quality requirements.²¹

Model	Function	Accuracy (AUC)	Strengths	Limitations
Deep Convolutional Neural Network	Extracts and processes attributes and image data using multiple layers. It was combined with clinical scores in the context of this study.	0.86 - DCNN & Mayo 0.84 - DCNN & Toronto	• Excels in processing image data • Automates attribute extraction from data	• Not very transparent, as coefficients aren't as interpretable compared to existing models. • Requires large datasets • Needs decent image quality to be effective

This study was limited by the fact that the sample size of 99 adults is relatively small, which has the potential to reduce its general usability. In addition to this, there is difficulty in understanding their classification reasoning. The coefficients used aren't always clear, making their reasoning and debugging processes increasingly more obscure. Grad-CAM provides some vague insight through deciding which input areas influenced the model's decision the most, but this lack of specificity could create a lack of transparency in the biases within the model's reasoning. The images used in the dataset were high quality, which may not be as generally applicable to hospitals with lower-end imaging. Genetic variant classifications may also change over time.

Machine Learning Models Predicting Muscle Hypertrophy Response:

The study by Yang *et al.* regard the use of three models: Generalized Linear Models (GLMs), Support Vector Machines (SVMs), and Random Forests (RFs). As directed in Figure 4, GLMs are flexible linear regressions that allow the use of response variables with non-normal error distributions, meaning they can process binary, count, and continuous data, as well as form distributions based on unusual error values (error distribution does not result in a normal distribution/bell curve). When deciding whether an individual was a responder/non-responder to Resistance or high-intensity training, a logistic regression GLM was used, functioning by taking a linear combination of input values such as Polygenic Predictor Scores (PPSs), age, and BMI, then using a logistic function to output a binary classifier between 0 and 1, representing the likelihood of someone being a responder, done through a logit link function. A logit link function takes in this linear combination, or sum, and then creates a positive odds value, in which the natural logarithm of all odds values is taken, creating a logit, in which the log of the odds of a class is then turned into a boolean value.¹⁴ GLMs are highly interpretable, with each feature having a coefficient that quantifies its influence on affecting the classification, as positive coefficients mean an attribute increases the probability of a specific classification occurring, and negative coefficients decrease this probability (Table 4).

The highest accuracy was obtained by the GLM approach with an AUC of 0.809. Moreover, it provided better interpretability. The SVM approach provided an accuracy of 0.792, which is capable of handling non-linear relationships. However, it is less interpretable and requires careful parameter tuning.

The Random Forest approach provided an accuracy of 0.695, which is robust to handling outlying values. However, it is less accurate and less interpretable. Further seen as SVMs, in Figure 4, find the optimal hyperplane to separate classes, handling non-linear relationships implicitly by mapping inputs in the high-dimensional spaces based on feature data, then, without computationally intensive complex equations, use support vectors to find the distance between the closest/accurate values to the boundary plane, which are seen as more important in determining a prediction. RFs create a collection of decision trees, allowing them to handle complex relationships between features without overfitting, but seem less interpretable than GLMs, as compared in Table 4.

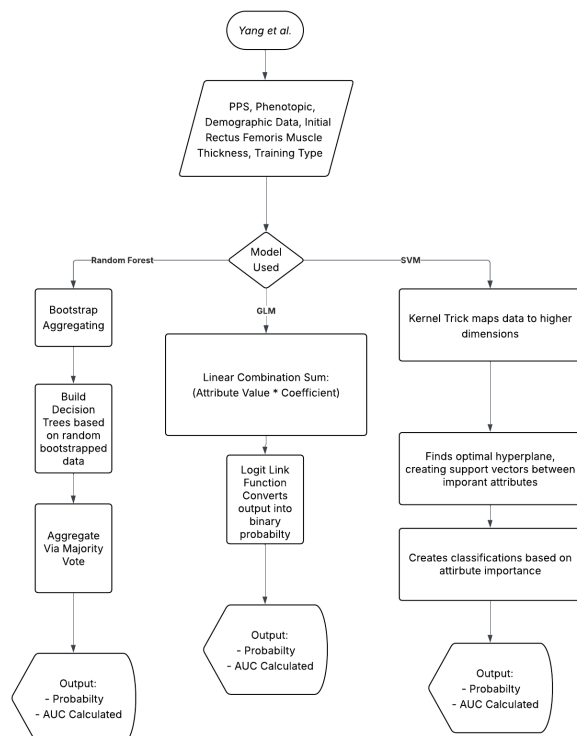


Figure 4: Process for Generalized Linear Models, Random Forests, and Support Vector Machines in predicting hypertrophy based on exercise type: Study on machine learning models in predicting hypertrophy across GLM, RF, and SVM.²⁴ Created using Lucid.

Table 4: Summary of Machine Learning Models for Predictive Analysis: Metric Comparison of GLMs, SVMs, and RFs in Hypertrophy prediction. Generalized Linear Models (GLMs) outperformed other models with an AUC of 0.809 for predicting skeletal hypertrophy caused by exercise, balancing accuracy with strong interpretability of feature coefficients.²⁴

Model	Function	Accuracy (AUC)	Strengths	Limitations
Generalized Linear Model	Creates a linear combination of inputs and a logit link function to create a boolean probability (between 0 and 1).	0.809	- Most accurate in the study - Highly interpretable coefficients	Assumes a linear relationship between the logit function's log-odds outputs and input features
Support Vector Machine	Ranks attributes/inputs by importance, removing the least important features through every iteration for optimization.	0.792	- Efficiently handles non-linear relationships well - Useful in high-dimensional spaces	- Less interpretable than GLM - Highly sensitive to parameter and kernel choices
Random Forest	Creates multiple decorrelated decision trees on random subsets of provided data and features, aggregated by voted majority.	0.695	Handles outliers efficiently	- Least accurate model in the study - Less interpretable/ More "Black Box-like" than GLM.

This study was limited in that the results generated from these models were derived from compound movements such as bench/squat and running intervals, reducing their universal exercise application. The sample size needed to be larger as well, as 450 participants is typically seen as a larger sample size for genome-wide association studies, which typically require millions of SNPs for higher applicability. Bias is present in the population, as they were all young, inactive Chinese adults, creating limitations for possibly identifying changes in hypertrophy across multiple habits and ethnicities. Lifestyle factors such as sleep and calorie intake, which play a large part in muscle growth, weren't measured as well, in addition to hormones associated with muscle growth, such as testosterone. It can be concluded from this study that GLM is the most efficient machine learning model for predicting hypertrophy in this context.

Skeletal Hypertrophy Analysis Using Random Forest Predictors:

Again, Random Forests (RFs) were used in Enzer *et al.*'s study, in which key additions were SHAP (Shapley Additive Explanations) values to explain each attribute variable's influence in determining a prediction, outlined in Figure 5. Based on game theory, SHAP assigns an importance value to an attribute for its contribution to a specific prediction. It calculates an attribute's marginal contribution by considering all combinations of features/attributes and their possible values, allowing for better interpretability to see which features are the most important.¹⁵ The use of SHAP analysis can help provide more clarity to models that typically lack it (contain a black-box nature), allowing them to be more ethically acceptable.¹⁶ These performance metrics are addressed in Table 5, which displays an AUC of 0.744 for the RF model, using SHAP analysis and K-means clustering to increase interpretability and allow for phenotypic subtyping, respectively. The model was further validated to identify this trend within multiple patient subtypes via K-Means clustering, identifying K number of patient subtypes through creating a clustered set of datapoints. The cluster's distance from all points within the dataset is then calculated, creating new clusters based on this calculated distance, assigning it to the nearest centroid, or point within that cluster, creating more clusters until each re-assignment presents little to no changes, creating groups based on data. This is seen in this study via attribute data such as race and age, showing a valid method in showing trends among a more specific group, given that the testing data initially presents little biases (Figure 5).¹⁷

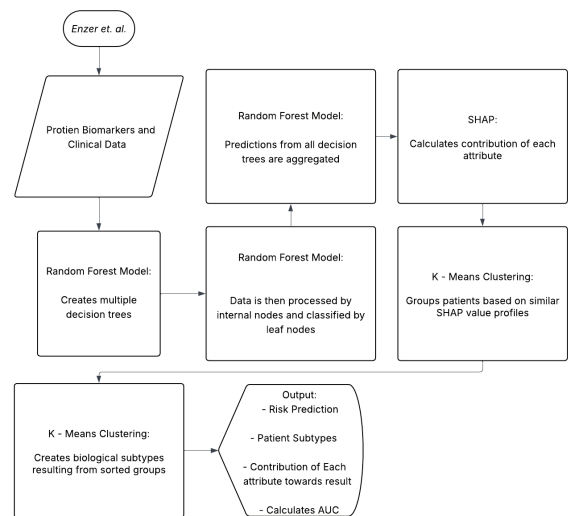


Figure 5: Process for Random Forest Models and Shapely Additive ExPlanations and K-Means Clustering model analysis: Study on machine learning models in predicting hypertrophy using RF models, then being analyzed using SHAP, then validated using K-means Clustering.²⁵ Created using Lucid.

Table 5: Summary of Machine Learning Models and Interpretability Methods: RF methods, and features of K-Means Clustering and SHAP Analysis in pattern analysis. While the Random Forest model had a moderate accuracy of AUC 0.744 for predicting sarcopenia development, integrating SHAP Analysis and K-means clustering was vital in improving interpretability and increasing the identification of patient subtypes.²⁵

Model	Function	Accuracy (AUC)	Strengths	Limitations
Random Forest	Creates multiple decorrelated decision trees on random subsets of provided data and features, aggregated by voted majority.	0.744	Handles outliers efficiently	- Least accurate model in the study - Less interpretable/ More "Black Box-like" than GLM.
SHAP Analysis	A Game-theory based method that calculates the contribution of each attribute to a prediction.	None	- Local and global model interpretability - Shows key drivers of prediction	Computationally expensive
K-Means Clustering	An unsupervised learning algorithm that creates groups/clusters based on the familiarity of SHAP values/data points.	None	Identifies phenotypic subtypes and patterns within the analyzed population	The number of clusters (k) can be subjective

Enzer *et al.*'s study utilizes machine learning to predict decreasing rates of hypertrophy in patients or the future development of sarcopenia. Using a Random Forest model, the data used to classify whether a patient had sarcopenia was derived from clinical data and eight protein biomarkers found in blood samples.

The limitations within this model are seen, as in its setup, it was trained on a specific group of smokers from a cohort in an ongoing study at the time, in which measurement also occurred less often due to a single CT Scan image being used to determine the initial muscle measurement and density. Overall, integrating ML appears to be a viable method of detecting changes in hypertrophy, even decreasing changes as seen in the case of sarcopenia, in which the validation methods used can help group smaller patient subclasses as well.

Molecular Pathways and Genes Associated with Muscle Hypertrophy:

Skeletal muscular hypertrophy occurs through microtears that occur in muscle, which then repair through processes such as sleep, increasing in size/thickness. This repair, mechanistically driven by resistance exercise and protein intake, activates the mTORC1 signaling pathway that regulates protein synthesis. Genes such as *Ski*, *Fst*, *Acvr2b*, *AKT*, and *Yap1* are more directly associated with hypertrophy.¹⁸ Illustrated in Figure 6, these molecular relationships are seen through anabolic and catabolic reactions from muscle tearing and their associated genes and pathways. These genes use the following pathways of Igf2-Akt-mTOR axis, myostatin-Smad pathway, and angiotensin-bradykinin pathway to increase anabolic reactions, promoting muscle growth from previously existing muscle, to trigger catabolic reactions to release energy and repress growth, and modulate hypertrophy, occurring the the Igf2-Akt-mTOR axis and the myostatin-Smad pathway respectively.^{18,19} Myostatin specifically is a negative regulator, in which its inhibition leads to muscle growth. *IGF1* and *Akt1* genes are also responsible for anabolic reactions in response to hypertrophic training. *Asb15* and *Tpt1* are also seen in skeletal muscle hypertrophy as well.²⁰

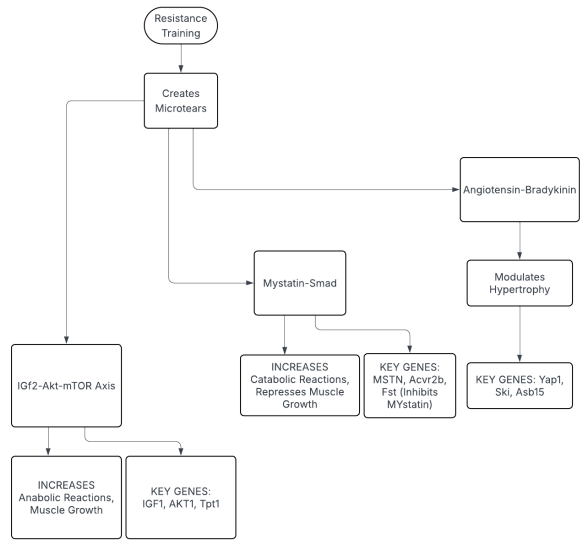


Figure 6: Process for Anabolic and Catabolic reactions from Muscle Tearing, and associated genes: "Gene-pathway diagram illustrating critical molecular pathways involved in skeletal muscle hypertrophy, highlighting genes such as *MYH6*, *FCN3*, *Ski*, *Fst*, and key signaling axes that influence muscle anabolism and catabolism.¹⁸

Discussion

In terms of results presented in each article seen in all studies, the model with the most accuracy in hypertrophy across both cardiac and skeletal hypertrophy is both of You and Dong's models, these being their SVM-RFE and LASSO models, respectively, having AUCs of 0.993 and 0.95, according to Table 6. Conversely, the ML model containing the lowest AUC was the RF model used in Enzer's idea, where its AUC is 0.695. Each study was conducted using a different validation method, as detailed in the Validation Method column of Table 6, including train/test splits and cross-validation, used in addition

to each study's respective machine learning model. Morita *et al.*'s model strengthened pre-existing prediction systems using Deep Convolutional Neural Networks, whereas Enzer *et al.*, on the other hand, used different types of data with the same RF model. In terms of the most efficient models in terms of AUC accuracy for models specializing in predicting skeletal hypertrophy, Yang *et al.*'s GLM had the highest AUC of 0.809. In contrast, its RF model had the lowest AUC of 0.695.

Table 6: Inter Model Comparison: In all, model performance varies by application, with SVM-RFE/LASSO accurate for gene identification with an AUC of 0.993, while the GLM is the most effective for exercise-induced skeletal hypertrophy with an AUC of 0.809. It further excels in accuracy/interpretability compared to the other models, making it the best for predicting exercise-based muscle growth, allowing for analysis on each attribute's impact in a classification.^{6,24}

Primary Focus	Model(s) Used	Key Predictors	Validation Method	AUC/Metrics
HCM Genotype Prediction	RF, GBDT, ADBT	Clinical parameters (LV wall thickness, FHx)	Train/Test Split	RF: 0.92, GBDT: 0.87, ADBT: 0.88
HCM Gene Biomarker ID	SVM-RFE, LASSO	mRNA expression levels	Cross-validated genomics	SVM-RFE (highest detected genes): 0.993, LASSO: 0.95
Genotype from Echo	Deep CNN (+ Mayo & Toronto scores)	Echocardiographic images + clinical models	5-Fold Cross-Validation	DCNN&Mayo: 0.86, DCNN&Toronto: 0.84
Exercise-Induced Hypertrophy	GLM, SVM, RF	PPS, Exercise Type, Baseline Metrics	10-Fold Cross-Validation	GLM: 0.809, SVM: 0.792, RF: 0.695
Sarcopenia Risk Prediction	Random Forest	Proteins (GDF15, EFEMP1), Clinical	Train/Test + Bayesian opt	Combined: 0.744, Biomarker: 0.740, Clinical: 0.646

This meta-analysis emphasises that predicting muscle hypertrophy requires a model that requires genomic information, proteomic and hormonal activity, as well as phenotypic and lifestyle baselines. Given each study, comparing the models used and their strengths, as well as their limitations, allows for a holistic model.

The studies on hypertrophic cardiomyopathy (HCM), even if not being targeted towards skeletal hypertrophy, are more focused on the predictive power of machine learning for genotype-affected outcomes, in which the models used could be applied to analyzing the predicted effect of several genes in skeletal hypertrophy. This could be seen in Liang *et al.* and Morita *et al.*'s studies, whose models were extremely accurate with AUCs reaching .92 and .88 via Random Forests (RFs) and Gradient Boosted Decision Trees in predicting sarcomeric mutations via clinical data.^{21,22} The Support Vector Machine-Recursive Feature Elimination (SVM-RFE) and LASSO regression were found to identify key gene biomarkers such as *MYH6* and *FCN3* accurately; these studies show that genetic architecture is a powerful predictor of muscular phenotypes as well, seen through their respective AUCs of 0.954 and 0.993 for determining the impact of these genes on muscular phenotype.⁶ Besides prediction, the models used in previous studies can be used to accurately gauge model growth, as a DCNN can also be used to measure muscle cross-sectional area from MRI scan data, ensuring training data is more accurate, rather than solely relying on muscle thickness.²³ While the application of ML in an HCM context provides direction, in terms of genetic effect on phenotype, the studies don't take into account direct factors that could affect muscle growth, such as exercise, diet, and sleep.

The lack of these aspects is then addressed in an all-around comparison of various machine learning algorithms, in which the Generalized Linear Model (GLM) outdid both the SVM and Random Forest (RF) models. This is due to GLM functioning as a flexible linear regression, where outcomes can also be binary count data based on the number of events, and multiple distribution structures. It assigns weights to multiple predictors, computing a linear combination of predictors to create a score that is then transformed through a logistic function to create a probability between 0 and 1 for that score, which is used to classify an output based on threshold/range conditions for probability.¹⁴ This impressive accuracy is seen in its AUC of 0.809, which, while not being the highest, was the most varied due to performance and interpretability, where its clear coefficients show that many polygenic predictor scores (PPN) and the associated exercise type (Such as resistance training or high interval intensity training) significantly affect predicted muscle growth.²⁴ It then becomes more eligible to be applied in real-world contexts of nutrition and medical advice. Prediction can be based on the thickness of a particular muscle group, in this case, the rectus femoris. This thickness, while not an exact indicator of strength, is an essential metric in measuring muscle growth itself.²⁴

Enzer's study takes a more protein-focused approach in predicting changes in hypertrophy via proteomics. For important biomarkers in showing a decrease in hypertrophy through detecting sarcopenia, their RF model was slightly less accurate than Yang *et al.*'s versatile GLM model (Table 6). This was seen, as it had an AUC of 0.740 for these biomarkers (Table 5), in which it identified eight protein biomarkers associated with muscle loss, such as GDF15 and EFEMP1²⁵ (Figure 5). Using SHAP analysis helped reduce its black box nature, showing the individual contribution of each predictor. Using k-means clustering on SHAP values to determine separate, smaller groups of patients based on phenotypic subtypes showed how different biologic classes in individuals can lead to similar results. Compared to previous models, the SHAP analysis and K-means clustering, which calculate the distance from the points selected to be the initial cluster/centroid, create more clusters to ensure accurate sorting into groups, which could be useful when taking in a variety of individuals, taking in factors such as age, race, genotypes, and hormone levels. In addition to evaluating significant attributes, ethical appeal is increased through its becoming easily understandable, making this model an Explainable AI, which would allow for easier implementation and stronger model trust in professional medical environments.²⁶

Given all these advantages over other algorithms, GLM serves as the most robust architecture, in which inputs can be polygenic risk scores summing the effects of hypertrophy-linked SNPs such as OR7A5, CCND3, and PARD3, as well as relevant hormone levels related to muscle growth, such as testosterone, IGF-1, and myostatin.¹⁸ In addition, initial muscle thickness, BMI, age, sex, sleep duration, and exercise should also serve as important parameters. Important biomarkers in hypertrophy detected in Enzer *et al.*'s study, such as GDF15, EFEMP1, CDON, Lymphotoxin a1/b2, VCAM-1, SPARC, Hat1, and NXPH1, were seen to indicate that dif-

ferent levels of these biomarkers would either show a higher or lower rate of developing muscle mass, in the low pectoralis muscle area specifically.²⁵ The consideration of these biomarkers is important, for example, GDF15. This biomarker acts as a stress-induced cytokine that restricts the atrophy, or shrinking of muscle tissue, seen in inflammatory conditions such as sarcopenia, and is increased by exercise. This is further seen as it plays a role as a stress-responsive cytokine released by muscle to respond to mitochondrial challenge. Induced by pathways such as the integrated stress response and mitochondrial unfolded protein response, it aids in regulating metabolism and energy balance.²⁷ Dividing data into a clear train/test split, implementing k-fold cross-validation, and performing SHAP analysis to reduce the model's unknown nature will increase its general usability and detection of smaller groups. Genotype and hormone levels create the foundational boundaries of possible muscle growth, while aspects such as phenotype and training methods create additional changes to these foundational muscle growth boundaries.

Furthermore, the model needs to be able to account for the fiber type and tissue specificity. It must take a specific approach tailored to a specific muscle/muscle group, in which the mass of the muscle shouldn't be the only differentiating factor in determining strength. Still, also functional outcomes for a more applicable model.²⁵ Additional relevant parameters should include robust phenotypic data, such as the fiber cross-sectional area, fiber type distribution, and functional strength to show whether growth improves movements. These requirements, focusing more on transparency in how it functions, may be difficult to include, given that the current majority of models, such as the ones evaluated, have a black-box nature, meaning their coefficients may not be as clear. Given this, Yang *et al.*'s study fulfills these requirements, focusing on a specific muscle group (rectus femoris) and using models that are highly transparent when compared to other models, such as GLM.²⁴

In terms of the studies seen and the models used, there is a greater need to include data relating to the direct hormonal effect on hypertrophy. Despite these hormones being important regulators of protein synthesis, the literature reviewed focuses more on genetics, proteomics, and cardiac imaging. This is likely due to the nature in which hormonal data is collected, as it requires constant sampling and complications in training models due to high biological variability. In terms of how these studies were set up, the participants were all in similar age/sex demographics, showing minimal hormone fluctuations, meaning the relationship between hormones and hypertrophy likely wasn't greatly prioritized in the scope of these studies. In terms of the direct impact of the inclusion of hormones such as testosterone levels and the IGF-1 gene on hypertrophy rates, as seen in Figure 6, these molecules result in anabolic reactions that then increase muscle growth through the Igf2-Akt-mTOR pathway. IGF-1 and increasing testosterone levels increase mechanical load-induced growth seen in skeletal hypertrophy, meaning that accounting for the rate at which hypertrophy is affected based on the involvement of key hormones and genes could help formulate a more complete gauge of one's potential muscle growth.

There are limits present in the actual execution of this model, as it raises ethical concerns. Initially, concerns are present in the individual's privacy, regarding whether the biological information being extracted from people can be kept safe, as well as the control certain experiments/procedures may have on an individual's life. This can be seen in ensuring someone follows a specific exercise, diet, and sleep routine daily over a prolonged period of time, in which extensive consent will be needed. While this model doesn't deal with PII such as credit cards and bank statements, attributes such as age and sex will be taken into account, meaning the use of this model should comply with legal guidelines. Taking into account AI in this context, the use of these ML models should entail descriptive processes of how each model within each study, or the proposed model used in this case uses patient data, highlighting a level of transparency within each model is important to highlight how the actual algorithms function, as well as how the algorithms specifically handle this PII and biodata as well. Any of these models shouldn't replace human expertise in treating or advising in these hypertrophy-related conditions, as they should be used as tools to help direct this assistance.

■ Study Limitations

Due to this study being a meta-analysis, it couldn't engage in empirical data collection, as it lacked all the data needed to ensure an accurate model. This is due to a lack of access to the technology needed to extract biodata from participants, as well as concerns surrounding a proper sample size. The latter is decided in the Events per Variable Rule (EPV), as according to Peduzzi *et al.*'s frequently cited study, *A simulation study of the number of events per variable in logistic regression analysis*, where the amount of data should range from the amount of events, or the necessary amount of rarer classes in this case, this being the amount of individuals with substantial response to hypertrophic actions, in which the amount of hypertrophic change/muscle thickness will decide how participants are classified.²⁸

There should be enough events for every variable being tested: these variables include phenotypic attributes, hormone levels, biomarkers, age, sex, as well as baseline muscle thickness and BMI. The amount of data needed can then be seen through the typical number of events (15), then multiplied by the 35 attributes/variables present, meaning a minimum of 525 events is needed.²⁹ This number of events is then multiplied by the amount of the rarer class's prevalence, this being 45% for those experiencing great positive change in hypertrophy, meaning that around 1575 (35×45) participants are needed to train a model, in the intention not to overfit data, meaning that a model isn't solely accurate for the training data.^{5,13} Given this great magnitude of individuals needed, the resources to acquire such a number of events aren't present, as well as low feasibility in convincing a diverse group of 1575 individuals. With the possible implementation of empirical data, the meta-analysis's findings could have been represented and applied more directly.

■ Conclusion

In all, taking into account several concepts and strengths from all the models analyzed in different hypertrophic contexts, a new framework is proposed based on the strengths and transparency present in the GLM model, as well as necessary parameters such as phenotypic data, genotypes, levels of protein biomarkers, initial muscle mass, and hormone levels. The model will use validation measures such as k-means clustering to identify smaller groups and subpatterns within the individuals assessed, with the intention of being geared specifically for separate muscle groups, meaning data gathered will have to be based on hypertrophy of a specific muscle. It was also discussed that this model should be used as a tool in addition to human guidance, as well as needing to be very transparent in its data and follow existing policies regarding individual health data, such as HIPAA. Its implementation stems further than simply collecting data, as when implemented directly, models trained on data of one demographic would overfit for that dataset, being inaccurate for broader use (algorithmic bias), in which the use of these overfitting models would create a large responsibility gap, as biased predictions may drive serious fitness decisions.³⁰

Furthermore, this study could be extended through the use of larger datasets, such as the application of said ML models discussed above in the context of muscle growth in multiple areas besides the lower pec and thigh areas, allowing for the accounting of more specific factors pertaining to hypertrophy trends in each muscle given certain activity. The inclusion of lifestyle variables such as alcohol/drug consumption, and how these additional substances play a part in regulating hypertrophy, could be addressed to account for dynamic changes an individual could experience while training, allowing for more educated control on the actions one may take in life.

What this study implies is that while various workflow processes are automated by existing AI technology, organic bodily processes that require both physical and mental effort can't be fully driven by AI or any rising technology for that matter. Increasing fitness interests across many signifies a need to accelerate the rate at which certain training plans are created, in which AI serves to bridge the gap between general fitness plans and users, as it can be used to optimize the specificity needed for efficient workout routines pertaining to the genetic makeup of an individual. Modern fitness needs to leverage AI's capabilities in creating optimized routines and lifestyle habits to promote an increasingly more athletic and well-developed population.

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Hydrothermal Synthesis of Bracken-Derived Carbon Quantum Dots: A Sustainable Approach for Efficient Ferric Ion Detection

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ABSTRACT: Biomass-derived carbon quantum dots (CQDs) have gained considerable attention as sustainable fluorescent materials due to their low toxicity, intense photoluminescence (PL), and facile synthesis. Among biomass sources, bracken, rich in polysaccharides, flavonoids, and organic acids, is a promising low-cost precursor for CQD production. In this study, we synthesize bracken-derived doped CQDs and explore their fluorescence (FL)-based sensing capability for ferric ions. Carbonized bracken powder was doped with sulfanilamide *via* a hydrothermal reaction, followed by purification of the resulting CQDs *via* a sequential filtration, centrifugation, and dialysis process. The CQDs were characterized *via* various instrumental analyses, such as FT-IR, UV-vis, and PL spectroscopy. In ion-sensing experiments, the effective adsorption of Fe^{3+} on CQDs induced pronounced fluorescent quenching, yielding a linear calibration curve in the range of 5–300 μM ($y = -0.35x + 174.14$). The limit of detection (LOD) and limit of quantification (LOQ) were calculated to be 8.52 and 25.80 μM , respectively. The results of the selectivity evaluation revealed that Fe^{3+} detection resulted in markedly greater fluorescent quenching than that of other metal ions. These results strongly demonstrate that bracken-derived CQDs function as effective and selective fluorescent probes for Fe^{3+} detection.

KEYWORDS: Environmental Engineering & Science, Green Chemistry, Carbon Quantum Dots, Metal Ion Adsorption, Fluorescence Sensing.

■ Introduction

In various environmental matrices, such as air, soil, and water, large quantities of heavy metals from industrial, agricultural, and urban activities can cause severe environmental pollution.^{1,2} While pollutants in water or air are transported and diluted by flow, heavy metals introduced into the soil become strongly adsorbed and fixed to soil particles, particularly clay minerals and organic matter.³ Furthermore, heavy metals entering aquatic systems are predominantly adsorbed onto particulate suspended matter rather than remaining in a dissolved state, eventually settling at the bottom of rivers and lakes. Unlike general organic chemicals or bacterial toxins, which can degrade over time or be detoxified by the immune system, heavy metals are elemental and thus non-biodegradable and persistent.⁴ Among heavy metals, ferric ion (Fe^{3+}) can be introduced into water systems at concentrations significantly higher than natural levels due to industrial activities such as acid mine drainage, factory wastewater, and metal corrosion.⁵ Because excessive Fe^{3+} disrupts aquatic ecosystems and contaminates drinking water sources, it is a critical target for detection in environmental monitoring networks. In addition, from a monitoring perspective, detecting Fe^{3+} is more practical for real-world environmental analysis, as Fe^{2+} is rapidly oxidized to Fe^{3+} —the final pollutant form—by atmospheric oxygen.⁶

The intense, tunable photoluminescence (PL) of quantum dots (QDs) enables them to serve as highly sensitive probes for detecting low concentrations of metal ions.^{7,8} Among various types of QDs, carbon quantum dots (CQDs) have received increasing attention because they are low-cost, non-toxic, photostable, and can be synthesized through eco-friendly “green

synthesis” routes.^{9,10} Therefore, the development of CQD-based fluorescence (FL) sensors for heavy metal ion detection addresses critical environmental monitoring needs while offering early-warning systems to safeguard human and ecological health. Recently, biomass has emerged as an important renewable carbon source with broad applicability.¹¹ Biomass-derived CQDs have been extensively studied due to their intense PL, photostability, catalytic properties, and low toxicity, which render them suitable for applications in sensors, catalysis, and bioimaging.¹² Furthermore, they offer distinct environmental advantages by promoting resource recycling and sustainable material development. Biomass can be readily converted into CQDs *via* various synthetic methods, such as hydrothermal treatment, microwave-assisted synthesis, and pyrolytic carbonization.¹³

Bracken (scientific name: *Pteridium aquilinum*) is a perennial fern with a ubiquitous global distribution. While it has historical significance as a dietary source in some regions, it is frequently categorized as an invasive and opportunistic weed in many Western areas, often requiring intensive physical or chemical removal. From a material science perspective, utilizing this abundant and underutilized biomass as a carbon precursor offers a sustainable and value-added upcycling strategy.

Bracken is a particularly effective biomass precursor for CQD synthesis due to its complex lignocellulosic framework. It is rich in cellulose, lignin, and a variety of phenolic compounds, which serve as excellent carbon and oxygen sources. During the hydrothermal process, these organic polymers undergo dehydration and carbonization, facilitating the formation of stable sp^2 -conjugated carbon domains that constitute the fluorescent

core of the CQDs. Furthermore, the presence of intrinsic heteroatoms and diverse functional groups within the bracken matrix promotes self-doping and surface functionalization, potentially enhancing the optical properties of the resulting nanoparticles without the need for excessive external reagents. Consequently, transforming this widely available invasive species into functional CQDs represents a cost-effective and eco-friendly approach to environmental monitoring.

In environmental monitoring, rapid and accurate detection technologies are indispensable. Although conventional methods, such as electrochemical sensors and mass spectrometry, offer high precision, they are often constrained by high costs, the requirement for skilled personnel, and time-consuming procedures. To address these limitations, FL-based sensing systems have emerged as a promising alternative, characterized by operational simplicity and high sensitivity.¹⁴ In this study, we synthesize N, S, and P-codoped CQDs utilizing carbonized bracken powder and sulfanilamide. Following the systematic characterization of their structural and optical properties, we evaluated their sensing performance, specifically targeting Fe^{3+} . Due to their paramagnetic nature and unfilled d-orbitals, Fe^{3+} ions act as effective electron acceptors and energy-transfer agents, serving as potent fluorescent quenchers. This interaction induces a pronounced quenching effect, thereby facilitating the highly sensitive and selective detection of Fe^{3+} .

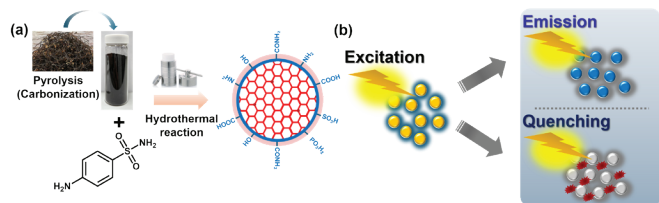


Figure 1: (a) Synthetic route of bracken-derived carbon quantum dots (CQDs) and (b) schematic illustration of PL emission (without any complex with ferric ions) and fluorescent quenching (by chelating with ferric ions) when CQD is photo-excited in an aqueous solution.

■ Methods

Materials:

Dried bracken, used as the carbon precursor, was sourced from Jeju, Republic of Korea, a region renowned for its abundant and high-quality wild bracken production. Sulfanilamide (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) was used as the N, S-dopant. Various metal salts were purchased to evaluate the ion-sensing selectivity of the CQDs: nickel(II) nitrate hexahydrate, sodium nitrate, and cobalt(II) nitrate hexahydrate, all obtained from Samjeon Pure Chemical Co., Ltd. (Pyeongtaek, Republic of Korea). Silver nitrate, magnesium sulfate, and potassium chloride were purchased from Duksan Pure Chemical Co., Ltd. (Ansan, Republic of Korea). Zinc nitrate hexahydrate, chromium nitrate nonahydrate, and barium nitrate were obtained from Daejung Chemicals & Metals Co., Ltd. (Siheung, Republic of Korea), while aluminum nitrate nonahydrate and iron nitrate nonahydrate were purchased from Junsei Chemical Co., Ltd. (Tokyo, Japan). All chemicals were used without further purification, and all organic solvents used were of ACS reagent grade.

Synthesis and Purification of Bracken-Derived CQDs:

Bracken-derived CQDs were synthesized as follows (Figure 1). Dried bracken was ground into a powder using a grinder. A 10 g sample of bracken powder was placed in a crucible and carbonized at 300 °C for 1 h in a furnace under an air atmosphere. The carbonized powder was finely ground and stored for further processing. The powder (3 g) was dispersed in 60 mL of distilled water, and a sulfanilamide solution (0.4 g) in 20 mL of ethanol was added. After stirring, the mixture was transferred to a hydrothermal reactor and heated at 200 °C for 4 h. The resulting solution was filtered through a 0.2 μm cellulose acetate membrane, and the filtrate was centrifuged at 13,500 rpm for 30 min using a Labogene mini centrifuge (Republic of Korea) to remove residual large particles. The supernatant was collected and dialyzed against distilled water for 24 h using a membrane with a molecular weight cut-off (MWCO) of 500 Da. During dialysis, the distilled water was replaced several times to ensure the removal of small impurities. The CQD solution was transferred from the dialysis tube into a round-bottom flask, and the solvent was removed using a rotary evaporator (IKA RV10, Germany). Finally, the CQD powder was collected from the round-bottom flask.

Structural and PL Characterization:

Surface functional groups and elemental composition were analyzed using Fourier transform infrared spectroscopy (FT-IR; FT/IR-6600, Jasco Co., Tokyo, Japan) and X-ray photoelectron spectroscopy (XPS; Nexsa, Thermo Fisher Scientific, Hillsboro, OR, USA), respectively. The crystalline structure was examined *via* X-ray diffraction (XRD; Panalytical Empyrean, Malvern Co., Malvern, Worcestershire, UK), while particle morphology and size distribution were characterized using transmission electron microscopy (TEM; Titan G2 ChemiSTEM, Thermo Fisher Scientific, Hillsboro, OR, USA). PL lifetime and absolute quantum yield (QY) were determined using a time-resolved fluorescent spectrometer (FLS-1000, Edinburgh Instruments, Livingston, Scotland, UK). The measurement was conducted using the absolute QY measurement method utilizing the equipped integrating sphere module. UV-vis absorption spectra were recorded with a Shimadzu UV-3600i Plus spectrophotometer (Kyoto, Japan), and PL emission spectra were obtained using a Jasco FP-6500 fluorometer (Tokyo, Japan).

Ferric Ion Detection:

Before the sensing experiments, FL stability was assessed under continuous 365 nm UV irradiation and across a pH range of 0.5–12.4. The effect of ionic strength on PL response was also investigated using varying KCl concentrations (0–1 M) to evaluate potential applicability in bioimaging. The Fe^{3+} sensing performance was examined by monitoring fluorescent quenching over a concentration range of 0–300 μM and calculating the detection sensitivity (limit of detection). Selectivity tests were performed using 0.5 mM solutions of interfering metal ions.

■ Results and Discussion

Structural Properties of Bracken-Derived CQDs:

The size and chemical structure of CQDs are critical factors in determining their fluorescent properties. As shown in the size distribution graphs (Figures 2a and 2b), dark grains of CQDs are well-dispersed, with an average particle size of approximately 9.30 nm. Given that this is larger than the typical size of CQDs (3–5 nm), this suggests that partial aggregation may have occurred. Moreover, as shown in Figure 2c, the CQDs display distinct lattice fringes across the entire particle area, indicating a graphitic-like crystalline structure. The d-spacing of the CQDs was measured to be 0.249 nm, corresponding to the (100) or (1120) plane (in-plane lattice of the hexagonal carbon ring in a graphene sheet).¹⁵ In the XRD pattern, a broad peak is observed around $2\theta = 21^\circ$, corresponding to a d-spacing of 0.42 nm. This indicates that the CQDs consist primarily of amorphous or turbostratic graphite components associated with the (002)-like plane, suggesting a disordered carbon framework. This structural characteristic may be attributed to the presence of surface functional groups and heteroatom doping, which will be further verified through FT-IR and XPS analyses in the following sections (Figure 3a).¹⁶

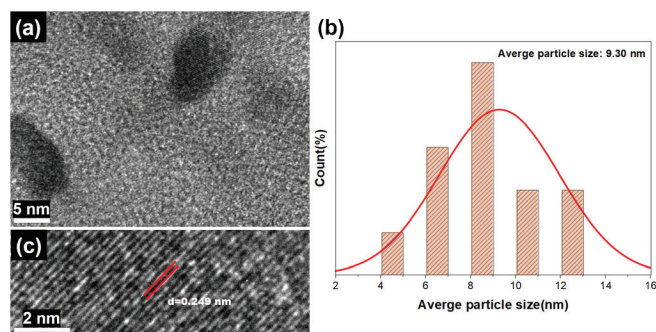


Figure 2: Morphology and structural characterization of the bracken-derived CQDs. (a) Transmission electron microscopy (TEM) image showing well-dispersed nanoparticles. (b) Particle size distribution histogram fitted with a Gaussian curve, indicating an average diameter of 9.30 nm. (c) High-resolution TEM (HRTEM) image displaying distinct lattice fringes with an interplanar spacing of 0.249 nm. TEM and HRTEM analyses of the bracken-derived CQDs show well-dispersed nanoparticles and lattice fringes.

The surface functional groups of the CQDs were further characterized by FT-IR spectroscopy. As shown in Figure 3b, a weak peak appears at 1640 cm^{-1} due to the stretching vibration of C=C bonds, which originate from the aromatic sp^2 (π -bonded) carbon domains within the CQD core. The absorption band at 3340 cm^{-1} is attributed to the overlapping vibrations of O–H and N–H functional groups.¹⁷ Additionally, the bending vibrations corresponding to N–H and C–N groups at 1570 cm^{-1} confirm the presence of nitrogen doping.¹⁸ Due to the sulfanilamide doping, symmetric and asymmetric stretching vibrations of SO_2 groups are observed at 1150 cm^{-1} and 1300 cm^{-1} , respectively.¹⁹ Peaks at 1100 cm^{-1} and 1450 cm^{-1} indicate the formation of C–O–P and P=O functional groups on the surface of the CQDs.²⁰ These functional groups likely originate from inorganic phosphate present in the bracken, which may condense into pyrophosphate species or form phosphate ester bonds with hydroxyl and carboxyl groups

during carbonization. Additionally, organic phosphate esters may decompose and dehydrate during the process, leading to the incorporation of P-doping sites on the CQD surface.

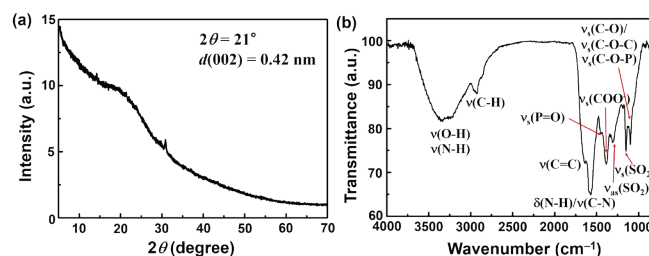


Figure 3: Spectroscopic characterization of the bracken-derived CQDs. (a) X-ray diffraction (XRD) pattern showing a broad diffraction peak centered at $2\theta = 21^\circ$, corresponding to an interlayer spacing (d-spacing) of 0.42 nm. (b) FT-IR spectrum identifying surface functional groups. XRD analysis reveals an amorphous or turbostratic carbon structure, while FT-IR spectra confirm the presence of N, S, and P-containing functional groups.

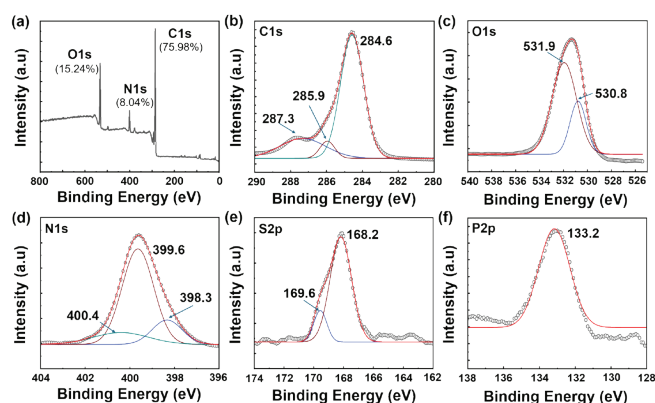


Figure 4: XPS analysis of the synthesized N, S, and P-codoped CQDs. (a) Full-scan survey spectrum showing the elemental composition. High-resolution spectra of (b) C 1s, (c) O 1s, (d) N 1s, (e) S 2p, and (f) P 2p. XPS analysis confirms successful N, S, and P codoping of the CQDs.

To further investigate these surface functional groups, the elemental composition and chemical structure of the N, S, and P-codoped CQDs were analyzed using XPS. As shown in Figure 4a, binding energy peaks corresponding to O1s, N1s, and C1s were observed at 545.0, 402.2, and 298.1 eV, respectively. Quantitative elemental analysis revealed that the CQDs contained 15.24 at% oxygen, 8.04 at% nitrogen, and 75.98 at% carbon. In addition, due to the introduction of dopants and the intrinsic components of bracken, small amounts of sulfur (0.59 at%) and phosphorus (0.14 at%) were incorporated into the CQDs.²¹ In other words, the resulting CQDs feature intentional N, S-doping and natural P-enrichment, inherited from the intrinsic phosphorus content ($\sim 0.2\%$ by dry weight) of the bracken precursor.²² In the high-resolution spectra (Figures 4b and 4c), three major peaks for carbon were identified at 284.6 eV (C–C/C=C), 285.9 eV (C–N/C–O), and 287.3 eV (C=O), while the oxygen peaks were mainly associated with C=O (530.8 eV) and C–O– (531.9 eV) bonds. These results are consistent with the FT-IR analysis, confirming that the CQD surface is rich in hydrophilic groups, including hydroxyl and carboxyl moieties.

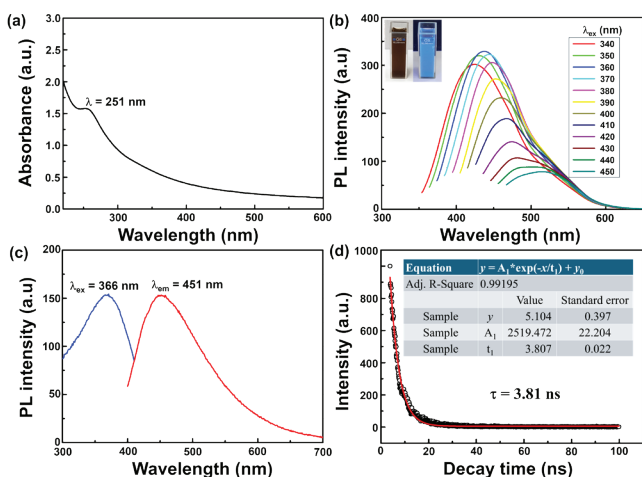


Figure 5: Optical properties of the bracken-derived CQDs. (a) UV-vis absorption spectrum showing a characteristic peak at 251 nm. (b) PL emission spectra were recorded under various excitation wavelengths from 340 to 450 nm. The inset displays photographs of the CQD solution under natural light (left) and 365 nm UV light (right). (c) PL excitation (blue line) and emission (red line) spectra, with maxima at 366 nm and 451 nm, respectively. (d) Time-resolved fluorescence decay profile fitted with a mono-exponential function, yielding an average lifetime (τ) of 3.81 ns. The CQDs exhibit a uniform radiative transition process with a peak emission at 450 nm.

Furthermore, the N1s spectrum (Figure 4d) clearly shows major peaks at 398.3 eV (pyridinic nitrogen), 399.6 eV (pyrrolic nitrogen), and 400.4 eV (graphitic nitrogen), indicating successful nitrogen doping as a key heteroatom. To investigate the minor dopants, the S2p and P2p spectra were analyzed (Figures 4e and 4f). Although present in trace amounts, distinct chemical states were observed. For sulfur, a peak corresponding to sulfoxide/sulfone (C-SO_x) was observed at 168.2 eV, while a sulfate ($-\text{SO}_4^{2-}$) peak appeared at 169.6 eV due to surface oxidation.²³ Additionally, the phosphorus signal showed a peak at 133.2 eV, attributed to P-O-C and P=O functional groups, which typically form during moderate-temperature synthesis (200–400 °C).

Optical Properties and Stability of CQDs:

An aqueous solution containing 0.1 mg/mL CQDs was used to investigate the optical properties of the CQDs. As shown in Figure 5a, the UV-vis absorption spectrum exhibits a single peak at 251 nm, which is typically attributed to the $n-\pi^*$ electronic transition of carbonyl (C=O) or nitroso (N=O) groups within the sp^3 -hybridized domains of the CQDs.²⁴ In addition, the inset photographs in Figure 5b display the appearance of the CQD solution under natural light (left) and UV light ($\lambda = 365$ nm). While the aqueous solution appears dark brown under natural light, it exhibits strong blue photoluminescence (PL) under UV irradiation. Given the average particle size from the TEM images, the blue emission likely arises from abundant surface defects and functional groups formed by the carbon precursor and dopant, rather than quantum confinement. Furthermore, the large particles observed in the TEM images may be characteristic of carbon polymer dots (CPDs), consisting of aggregates of individual blue-emissive CQDs. As the excitation wavelength (λ_{ex}) is varied from 340 to 450 nm, the emission peaks shift from 420 to 520 nm (Fig-

ure 5b). The highest PL intensity was observed at an excitation wavelength of 360 nm. This characteristic behavior of CQDs is primarily determined by variations in particle size and the distribution of emission trap sites.²⁵

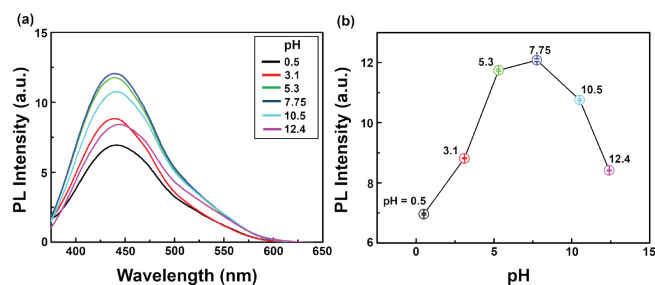


Figure 6: Effect of pH on the PL intensity of the bracken-derived CQDs. (a) PL emission spectra were recorded at different pH values ranging from 0.5 to 12.4. (b) Variation of the maximum PL intensity as a function of pH, indicating the pH-dependent fluorescence behavior. This result suggests that the PL intensity is stable near neutral pH but drops at extreme pH levels due to surface group changes.

To investigate the PL properties in more detail, PL excitation (PLE) and emission spectra were obtained. As shown in Figure 5c, the PLE spectrum exhibits an absorption peak at 366 nm, corresponding to electron transitions from the highest occupied molecular orbital (HOMO) to the lowest unoccupied molecular orbital (LUMO) within the π -orbitals (or sp^2 domains). Upon excitation at this wavelength, the maximum emission peak appears at 451 nm, with a full width at half maximum (FWHM) of approximately 125 nm. Furthermore, the fluorescence lifetime of the CQDs was determined to be 3.81 ns based on the FL decay profile measured at an excitation wavelength of 370 nm and an emission wavelength of 450 nm (Figure 5d). This single-exponential decay behavior indicates a relatively uniform radiative transition process in the CQDs.²⁶ In addition, the fluorescent QY under 370 nm UV excitation was measured to be 0.03% in an aqueous solution and 0.19% in a 1:1 (v/v) ethanol/distilled water mixture. The PL emission of these CQDs is attributed to the radiative recombination at passivated surface energy traps.²⁷

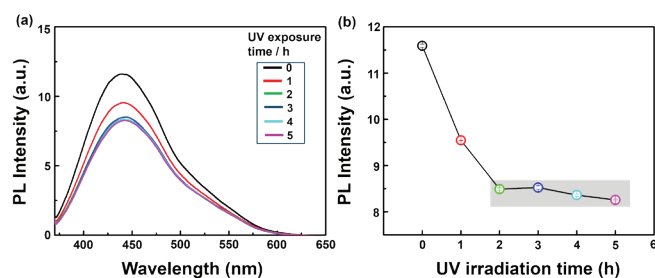


Figure 7: Photostability evaluation of the bracken-derived CQDs under continuous UV irradiation. (a) PL emission spectra recorded at 1-h intervals over a period of 5 h ($\lambda_{\text{ex}} = 365$ nm). (b) Time-dependent variation of the PL intensity. The shaded region indicates the stabilization of PL emission after 2 h of UV irradiation. This result shows that the emission stabilized under prolonged UV irradiation, following the initial 2 h decline caused by UV degradation.

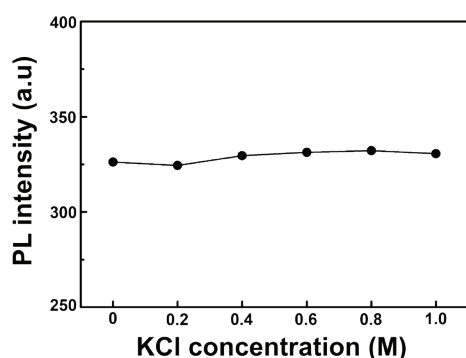


Figure 8: Stability analysis of CQDs (0.1 mg/ml) via fluorescence emission spectra under varying ionic strengths (0–1 M KCl).

To determine the optimal operating conditions for the sensor, the effect of pH on the PL intensity of the CQDs was investigated over a wide pH range (0.5–12.4). As shown in Figure 6a, the PL intensity shows a strong dependence on solution pH. The corresponding trend in Figure 6b clearly indicates that the PL emission reaches its maximum at pH 7.75 and remains relatively stable near neutral conditions (pH 5.3–7.75). However, a significant decrease in PL intensity is observed under strongly acidic (pH < 3) or alkaline (pH > 10) conditions. This phenomenon can be attributed to the protonation and deprotonation of the abundant surface functional groups (e.g., $-\text{COOH}$, $-\text{NH}_2$, $-\text{OH}$). In highly acidic environments, protonation of carboxyl and amino groups can induce CQD aggregation or alter their surface electronic states, leading to fluorescent quenching.²⁸ Conversely, under strongly basic conditions, the deprotonation of surface moieties can facilitate non-radiative electron recombination pathways, resulting in reduced emission. The high stability observed near neutral pH suggests that the synthesized CQDs are well-suited for environmental and biological applications that require physiological pH conditions.

Additionally, to evaluate the fluorescent stability of the CQDs, the emission intensity was measured at 1-h intervals over 5 h under continuous UV irradiation ($\lambda_{\text{ex}} = 365 \text{ nm}$) (Figure 7a). Although the emission intensity decreased gradually over time, it retained 71.2% of its initial value. During the initial 2 h, the emission decreased significantly due to UV degradation; however, the emission stabilized upon prolonged UV irradiation (Figure 7b), demonstrating UV photostability. Therefore, the CQDs exhibit excellent long-term fluorescent stability and low susceptibility to degradation, indicating strong potential for practical applications. It is noteworthy that the fluorescence degradation occurred primarily during the initial 2 h. This initial drop can be attributed to the photo-oxidation or photobleaching of thermally unstable functional groups or surface defects on the CQDs.²⁹ However, after this "burn-in" period (0–2 h), the PL intensity reached a steady state (indicated by the grey shaded region), showing negligible further variations. This suggests that the core structure and stable surface passivation layers of the CQDs are robust against prolonged UV exposure. Consequently, despite the initial attenuation, the CQDs exhibit sufficient long-term photostability to serve as reliable fluorescent probes. Further-

more, the fluorescence intensity of CQDs was monitored in aqueous KCl solutions with various concentrations ranging from 0 to 1 M (Figure 8). Across the entire concentration range, the change in fluorescence intensity ($I_{\text{PL,ave}} = 329.1 \pm 3.1$ at $\lambda_{\text{ex}} = 360 \text{ nm}$, $\lambda_{\text{em}} = 436 \text{ nm}$) was negligible. This result indicates that the CQDs exhibit excellent stability even under high-salt conditions, suggesting their strong potential for various applications in complex and harsh environments.

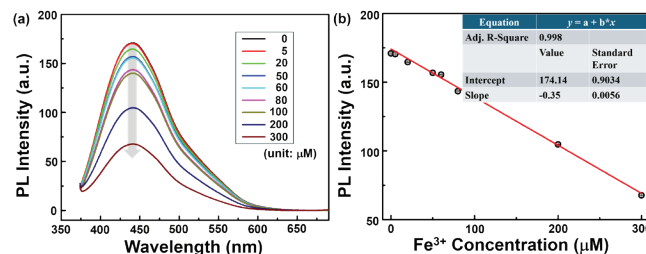


Figure 9: Fluorescence sensing performance of the bracken-derived CQDs toward Fe^{3+} ions. (a) PL emission spectra of the CQDs (0.1 mg/mL) in the presence of various Fe^{3+} concentrations ranging from 0 to 300 μM . (b) Linear calibration plot of PL intensity versus Fe^{3+} concentration. This result displays that PL excited at 360 nm showed a gradual decrease in emission intensity at 440 nm as the Fe^{3+} concentration increased.

Metal Ion Sensing Properties:

In general, Fe(III) ions induce interparticle crosslinking/aggregation through mono- or bidentate chelation with carboxylate ($-\text{COO}^-$) groups present on the surface of the CQDs. For the principal dopant nitrogen (N), monodentate coordination can occur *via* $-\text{NH}_2$; moreover, cooperative interactions between NH_2 and $-\text{COO}^-/\text{C}=\text{O}$ groups can lead to the formation of mixed O,N-chelation complexes.^{30,31} Additionally, for trace sulfur doping, $-\text{SO}_x$ can strongly coordinate with Fe^{3+} , and adjacent $-\text{S}-$ together with $-\text{COO}^-/-\text{OH}$ can bind simultaneously to produce multidentate chelates.³² In the phosphorus-doped regions, bidentate coordination (two O atoms binding to a single Fe^{3+}) is also feasible.

Based on these various coordination possibilities, the concentration dependence of adsorption was investigated by monitoring PL attenuation of a CQD solution (0.1 mg/mL) upon exposure to Fe^{3+} over 5–300 μM (Figure 9a). As the Fe^{3+} concentration increased, PL excited at 360 nm showed a gradual decrease in emission intensity at 440 nm. As shown in Figure 9b, a linear calibration curve, $y = -0.35x + 174.14$, obtained over 5–300 μM yielded a sensitivity of $0.35 I_{\text{PL}}/\mu\text{M}$ and an R^2 of 0.998. Using this calibration, the limit of detection (LOD) and limit of quantitation (LOQ) were calculated as 3.3 ($S/|m|$) and 10 ($10S/|m|$), respectively, where S is the standard deviation of the y-intercept and $|m|$ is the slope of the regression line.³³ The corresponding values were 8.52 μM for LOD and 25.80 μM for LOQ.

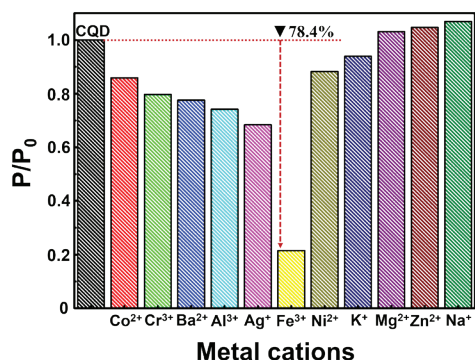


Figure 10: Selectivity evaluation of the bracken-derived CQDs toward various metal ions. The bar chart displays the relative PL intensity (P/P_0) in the presence of different metal ions (0.5 mM). PL intensity at $\lambda_{em} = 440$ nm was collected under UV light ($\lambda_{ex} = 360$ nm) after the adsorption of each metal ion in CQD solutions (0.1 mg/mL). This result suggests that Fe^{3+} ions induced a distinct fluorescent quenching effect compared to other metal ions.

Specific oxygen-containing functional groups on the surface of CQDs can serve as selective coordination sites for transition-metal ions, making them promising candidates for ion detection. As shown in Figure 10, to investigate PL intensity changes caused by metal ion adsorption, a doped CQD aqueous solution (0.1 mg/mL) was prepared, and various metal ions (0.5 mM) — Co^{2+} , Cr^{3+} , Ba^{2+} , Al^{3+} , Ag^+ , Fe^{3+} , Ni^{2+} , K^+ , Mg^{2+} , Zn^{2+} , and Na^+ — were introduced to evaluate selective adsorption behavior. Among them, Fe^{3+} ions induced a distinct fluorescent quenching effect compared to other metal ions. Specifically, the PL intensity decreased by approximately 78.4% relative to the baseline of the CQDs upon Fe^{3+} adsorption. In contrast, the PL responses for Co^{2+} , Cr^{3+} , Ba^{2+} , Al^{3+} , Ag^+ , Ni^{2+} , and K^+ ions remained within the range of 68–88% of the initial value. Furthermore, in the presence of Mg^{2+} , Zn^{2+} , and Na^+ ions, the PL intensity exhibited almost no change, indicating that the synthesized CQDs were not affected by the adsorption of these ions.

■ Conclusion

In this study, we successfully synthesized N, S, and P-co-doped CQDs *via* a facile hydrothermal route, using bracken, an abundant biomass, as a green carbon precursor and sulfanilamide as a dopant. The structural analyses (TEM, XRD, XPS, and FT-IR) confirmed that the CQDs possess a graphitic core with an average diameter of 9.30 nm and are rich in hydrophilic surface functional groups ($-COOH$ and $-OH$) and heteroatoms (N, S, and P). These surface states endowed the CQDs with excellent water solubility and stable blue PL centered at 451 nm. The CQDs exhibited remarkable stability against high ionic strength and long-term UV irradiation, highlighting their suitability for practical sensing applications. Notably, the CQD-based sensor exhibits stable and high sensitivity within the pH range of 5.30–7.75. This range is particularly significant as it encompasses the physiological pH of biological fluids and the typical pH levels found in natural water bodies. The ability to detect Fe^{3+} within this near-neutral window without extensive pH adjustment highlights the practical utility of the proposed probe for real-time environmental monitoring and

bio-analytical applications. In sensing experiments, the CQDs demonstrated high selectivity and sensitivity toward Fe^{3+} , attributed to strong fluorescent quenching induced by Fe^{3+} coordination to surface carboxyl, amine, and doped functional groups. The sensor showed a wide linear response range of 5–300 μM with the LOD of 8.52 μM . Despite these promising results, a current limitation of this study is the relatively low QY (0.03%), which may restrict the sensor's performance in environments with high optical background noise. To address this, our future research will focus on optimizing the hydrothermal reaction parameters and exploring advanced surface passivation techniques to enhance the radiative recombination rate. Furthermore, validation in complex environmental matrices, such as tap and river water, remains a priority to assess the practical robustness of the probe. Consequently, this work suggests that converting bracken into functional CQDs is a promising, eco-friendly strategy for developing low-cost fluorescent probes for environmental monitoring of heavy metal ions.

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Natural Genetic Variation in Mitochondrial Health-Regulating Genes in the *C. Elegans* Strains CX11314 and EG4725 Leads to Increased Mitochondrial Resilience.

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ABSTRACT: Mitochondria play a central role in maintaining cellular function and homeostasis, and their dysfunction can severely impact lifespan and aging. Owing to their conserved pathways, human orthologs, and experimental tractability, *Caenorhabditis elegans* was used as the model organism in this study. We investigated natural genetic variation in the *C. elegans* strains CX11314 and EG4725, which are characterized by increased resistance to mitochondrial dysfunction. Specifically, we examined polymorphisms and differential expression in six key mitochondrial health-regulating genes—*atfs-1*, *eat-3*, *fzo-1*, *pdr-1*, *drp-1*, and *pink-1*—that control mitochondrial morphology and mitophagy, using variant analysis, phylogenetic comparison, and RNA-seq data. Our analyses identified strain-specific SNPs in these genes and revealed differences in their expression levels, suggesting that natural genetic variation may modulate mitochondrial resilience. Building on these findings, we propose applying CRISPR-Cas9-mediated homology-directed repair (HDR) genome editing to directly test how specific variants in mitochondrial-regulating genes give rise to distinct phenotypic and behavioral outcomes.

KEYWORDS: *C. elegans*, Mitochondrial Dysfunction, Mitophagy, Mitochondrial Morphology, Mitochondrial Resilience.

■ Introduction

According to the Cleveland Clinic, approximately 1 in every 5,000 individuals is affected by a genetic mitochondrial disease.^{1,2} These diseases can affect nearly every organ, ranging from the brain and nervous system to the heart and liver, emphasizing the importance of analyzing these conditions through the various related genes and genetic pathways.

Its transparent body allows scientists to easily observe internal structures and track subtle changes in development, behavior, and gene expression with ease.³ Its short lifespan helps researchers quickly observe the effects of genetic or environmental changes across an entire lifespan, making experiments more efficient. Additionally, since *C. elegans* are hermaphrodites, they will be able to produce offspring through self-fertilization. This minimizes genetic variation in the tests since all offspring will be genetically identical across generations. Furthermore, researchers can directly translate discoveries made with *C. elegans* to human diseases, as there are conserved genetic pathways and functional orthologs.

It is crucial to note that varying *C. elegans* strains should be used, as the majority of studies pertaining to *C. elegans* have been conducted on the N2 (Bristol) strain.⁴ However, conducting research on strains with unique abilities like heteroplasmy resistance will reveal the genetic variations that result in that characteristic. Therefore, this study will focus on two *C. elegans* strains that have shown improved mitochondrial function and resilience: CX11314 and EG4725. By identifying genes and expression pathways that contribute to their improved mitochondrial morphology and mitophagy, we aim to discover

novel insights into the biological mechanisms that promote healthy aging. These discoveries will then have the potential to facilitate research into age-related decline in humans.

Previous research has found that mitochondrial dysfunction accumulates as individuals age, and removing functionally compromised mitochondria helps organisms recover from the adverse effects.⁵ In fact, research in fruit flies has shown that by removing damaged mitochondria, male and female fruit flies were able to live 20 percent and 12 percent longer, respectively. Additionally, previous studies have discovered cellular mechanisms that maintain mitochondrial health and slow down the aging process. In the N2 *C. elegans* strain, mitochondrial fission genes (*drp-1*), fusion genes (*fzo-1* and *eat-3*), mitophagy genes (*pink-1* and *pdr-1*), and mitochondrial unfolded protein response (UPR) genes (*atfs-1*) have been identified. These findings have identified that these genes assist in maintaining mitochondrial structure, responding to stress, and removing damaged mitochondria.⁶⁻⁸ However, when these processes fail, cells accumulate dysfunctional mitochondria, contributing to aging, neurodegeneration, and other chronic diseases.⁹ As mitochondrial dysfunction continues to emerge as a key factor in many human diseases, understanding the genetic control of mitochondrial maintenance is more relevant than ever. Yet, despite these advances, most research relies on lab-adapted strains and overlooks the natural genetic diversity that could reveal new, potentially therapeutic, pathways to promote mitochondrial resilience and healthy aging. As shown in Figure 1, the protein uaDf5 shows varied expression across the wild isolate strains, indicating significant diversity in protein levels.

Future studies are also focusing on studying the evolutionary adaptations and genetic differences between different strains of *C. elegans*. This shift from single-strain to multi-strain comparisons can help researchers uncover many evolutionary adaptations in *C. elegans* that are caused by the different environmental pressures. In a study by Zhang *et al.*, researchers analyzed gene expression across 207 wild *C. elegans* isolates and showed that natural diversity in gene expression and possible regulatory mechanisms highlight the promise of using gene expression variation to understand how phenotypic diversity is generated.¹⁰ Furthermore, recent research projects have started to use natural genetic variation in *C. elegans* strains as a tool for discovering new aging regulators. For example, a paper by Yin *et al.* discusses how different *C. elegans* isolates show diverse lifespans and age-related declines. In the study, they identified natural genetic factors that influenced the rate of aging in *C. elegans* by looking at the different aging mechanisms among the different *C. elegans* isolates and comparing them to their genetic variance.¹¹

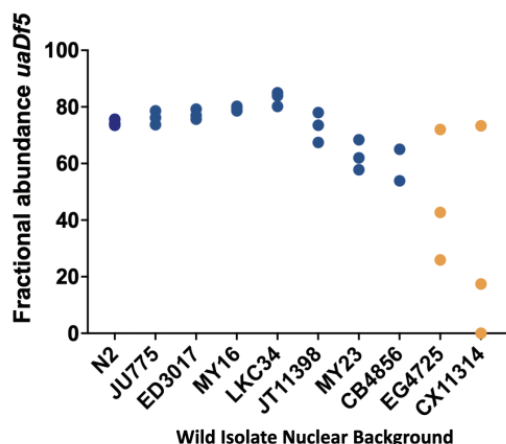


Figure 1: The fractional abundance of the protein uaDf5 for each wild isolate strain. This figure is based on unpublished data provided by the Rothman Laboratory at UCSB. This variation highlights substantial diversity in mitochondrial-related protein abundance among wild *C. elegans* strains.

The field of aging genetics in *C. elegans* has directed its focus on mito-nuclear interactions and post-transcriptional control under stress. Recent studies have also found that these interactions regulate mitochondrial function and aging. Furthermore, lifespan is a polygenic trait heavily influenced by approximately 293 nuclear regions as well as widespread mito-nuclear interactions, with worms with enhanced interactions living significantly longer.¹² In recent years, studies have been utilizing gene expression data for analysis. Unfortunately, there remains a major gap in understanding how SNPs and varying gene expression improve mitochondrial health. As illustrated in Figure 2, mitochondrial quality control involves coordinated regulation of nuclear and mitochondrial genes governing fusion, fission, mitophagy, and stress response.

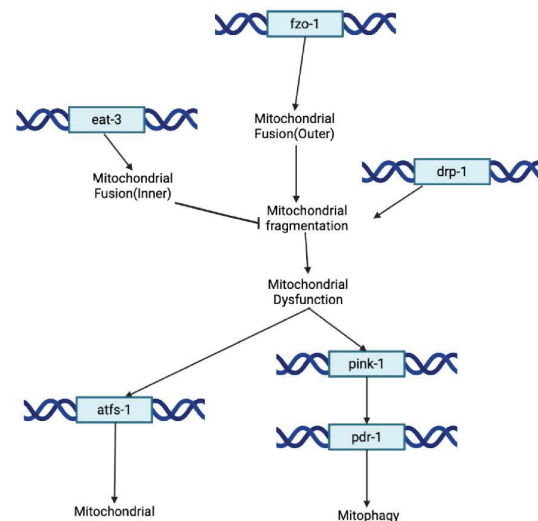


Figure 2: Overview of mitochondrial quality control pathways with mitochondrial and nuclear genes. These pathways collectively maintain mitochondrial integrity and cellular homeostasis.

This leads to our research question: What genetic variants in mitochondrial morphology and mitophagy-related genes contribute to the regulation of mitochondrial quality and heteroplasmy in the *C. elegans* strains CX11314 and EG4725 compared to the N2 reference strain? We hypothesize that genetic variation and variable expression in mitochondrial morphology and mitophagy-regulating genes (*drp-1*, *fzo-1*, *pink-1*, *eat-3*, *pdr-1*, and *atfs-1*) enhance mitochondrial quality control in the wild *C. elegans* strains CX11314 and EG4725, leading to reduced mitochondrial dysfunction and lower levels of heteroplasmy compared to the N2 reference strain. Our study aims to answer these questions by analyzing genomic variants and expression levels through the utilization of bioinformatics. As depicted in Figure 3, our methodology combines variant analysis, gene expression analysis, and functional testing to assess mitochondrial health in the strains.

Methods

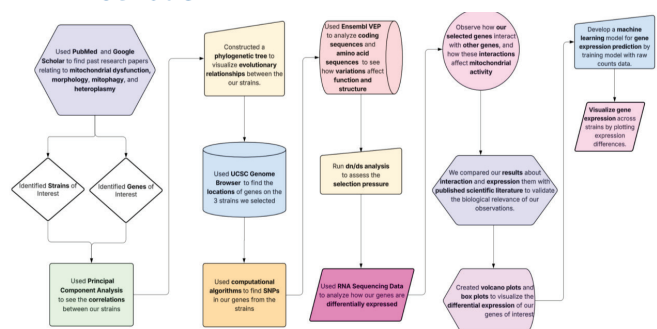


Figure 3: Methodological workflow used in this study. The workflow includes literature-based gene selection, Caenorhabditis elegans variant analysis, PCA and phylogenetic comparison, VEP and dN/dS analyses, RNA-seq expression analysis, machine-learning-based EG4725 expression prediction, and proposed CRISPR-Cas9 functional validation.

To investigate the genetic differences underlying mitochondrial function in *C. elegans* strains, we designed a stepwise bioinformatics workflow that combined literature review, variant analysis, phylogenetic comparison, differen-

tial gene expression analysis, and functional interpretation. First, PubMed, PMC, Europe PMC, and Google Scholar were searched for studies related to mitochondrial morphology, mitophagy, mitochondrial dysfunction, and heteroplasmy. Based on this review, we selected the N2 reference strain and the CX11314 and EG4725 strains for further analysis because CX11314 and EG4725 have been associated with improved mitochondrial function compared with the standard N2 strain. We then focused on six genes involved in conserved mitochondrial quality-control processes: *atfs-1*, *eat-3*, *fzo-1*, *pdr-1*, *drp-1*, and *pink-1*. Comparing these strains with N2 allowed us to identify genetic and expression-level differences that may contribute to mitochondrial resilience.

After obtaining Variant Call Format (VCF) files from Caenor, a *C. elegans* natural diversity resource, bioinformatics Python libraries such as scikit-allel (v1.3.13), Biopython (v1.85), ETE3 (v3.1.3), PyDESeq2 (v1.40.2), NumPy (v2.3.2), Matplotlib (v3.10.8), and SciPy (v1.16.0) were utilized for further analysis.^{13–20} Principal Component Analysis (PCA), a statistical method commonly used to simplify complex, high-dimensional data while preserving variance, was used to compare variants between 540 isotypes. This visualization helped assess the genetic similarities, where the distance between each strain increases with genetic divergence.

A phylogenetic tree was then used to visualize the overall evolutionary relationships and genetic distances among the 12 selected *C. elegans* isotypes: MY1, AB1, EG4725, CB4856, N2, MY16, JU393, ED3017, DL238, LKC34, GXW1, and CX11314. The UCSC genome browser, an online tool that allows scientists to analyze DNA from different organisms, was used to find the locations of our selected genes in the *C. elegans* genome. The gene ranges obtained from the genome browser helped us identify genetic differences in our strains of interest by consulting the VCF data.

To identify differentially expressed genes, we processed RNA-seq counts data for DESeq2 analysis, comparing the CX11314 and N2 strains. Using the VCF data, we identified SNPs where the strains differed and generated variant tables, helping us pinpoint mutations that could affect gene function and explain expression differences from our RNA-seq results.

The Ensembl Variant Effect Predictor (VEP) was used to compare DNA, RNA, and protein sequences and identify nucleotide and amino acid differences.²¹ This analysis helped us examine how genetic variants may affect protein structure and function, thereby influencing mitochondrial health. To determine whether the observed genetic differences in the genes of interest were likely shaped by selection, the nonsynonymous/synonymous substitution ratio (dN/dS) was calculated for each candidate gene.

Gene expression data from 609 samples across 208 strains were obtained from the NCBI GEO dataset GSE186719, which includes raw read counts and TPM (Transcripts per million) values.²² These data were used to examine expression differences in mitochondrial-related genes between CX11314 and N2 and to approximate EG4725-related expression patterns. Transcript isoforms were identified using WormBase, which provides transcript names, genomic coordinates, and

functional annotations. Differential expression analysis was performed using PyDESeq2 on raw read counts, while TPM values were used for visualization. Because EG4725 was not included in GSE186719, NIC266 was selected as a proxy strain based on genetic similarity. Earlier PCA analysis of 540 isotypes identified NIC266 as the closest strain to EG4725, and VCF analysis showed very few SNP differences between NIC266 and EG4725. Therefore, RNA-seq expression data from NIC266 were used in place of EG4725 for differential expression analysis and visualization, while this limitation was clearly considered during interpretation.

Following the DESeq2 analysis, differentially expressed genes were visualized using volcano plots and box plots. Volcano plots highlighted genes with statistically significant fold changes using a Benjamini-Hochberg (BH) adjusted p-value threshold of < 0.05 .²³ Box plots generated from TPM values showed the distribution of expression levels for selected genes across N2, CX11314, and NIC266. Because direct RNA-seq expression data for EG4725 were unavailable, a machine-learning approach was used to predict its gene expression profile. After data cleaning, a neural network with leave-one-out cross-validation (LOOCV) was developed; NIC266 was left out during validation because it is genetically closest to EG4725. The model was implemented using Python packages including Keras (v3.10.0), TensorFlow (v2.19.0), scikit-learn (v1.7.1), and SciPy (v1.16.0). Spearman's correlation was then used to evaluate the confidence of the EG4725 expression prediction.²⁴ Finally, gene interaction patterns were reviewed and cross-referenced with existing literature to interpret the biological relevance of the expression results.

■ Results

CX11314 and EG4725 Are Genetically and Evolutionarily Divergent from N2:

The genetic divergence among the *C. elegans* strains is highlighted in Figures 4 and 5, where PCA shows the clustering of the strains based on genetic similarities.

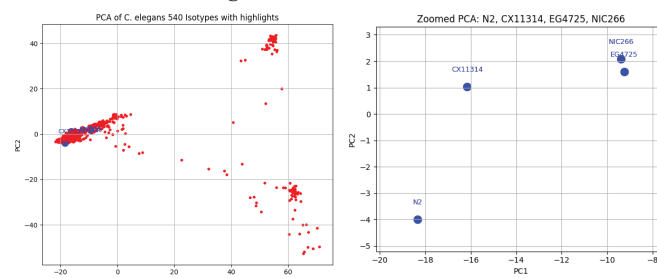


Figure 4: Principal Component Analysis (PCA) depicting the clustering of genetically similar of interest. The zoomed-in view of *C. elegans* strains. This clustering emphasizes the relative genetic distance of CX11314 and EG4725 among strains based on SNP variation.

A principal component analysis (PCA) was then utilized to visualize the genetic distance between the 540 different *C. elegans* strains using the SNP data. In both Figures 4 and 5, the data points represent N2, CX11314, and EG4725. Additionally, as the gene expression data for EG4725 was limited,

NIC266, the strain genetically closest to EG4725, was used as a proxy. The evolutionary relationships among the strains are visualized in Figure 6, providing insights into their genetic distance.

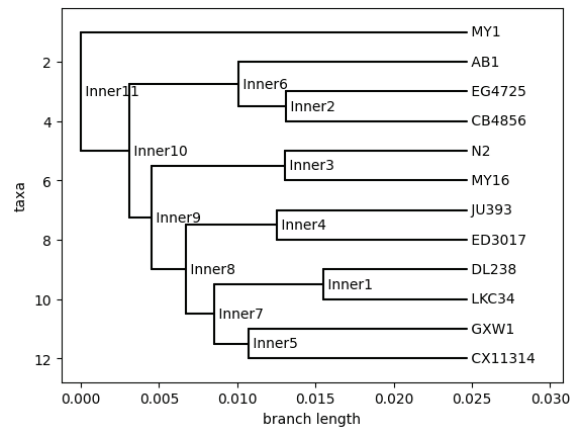


Figure 6: Phylogenetic tree showing evolutionary relationships in different *C. elegans* strains. This tree confirms that CX11314 and EG4725 diverged earlier from the N2 lineage.

The phylogenetic tree based on SNP data further illustrated the evolutionary relationships among the selected strains. Figure 6 shows that CX11314 and EG4725 are both genetically and evolutionarily distinct from N2. For example, the last common ancestor between N2, CX11314, and EG4725 was located at the Inner10 node, suggesting that these strains did not diverge recently.

Purifying and Neutral Selection in Genes:

Table 1: Ratio of non-synonymous to synonymous mutations for respective genes. These results indicate that several mitochondrial genes are evolutionarily conserved under selective pressure.

Gene	dN	dS	dN/dS	Significance
atfs-1	34	41	0.829	Purifying
pink-1	5	5	1	Neutral
drp-1	12	20	0.6	Purifying
pdr-1	15	19	0.789	Purifying
eat-3	5	5	1	Neutral
fzo-1	5	5	1	Neutral

Table 1 presents the dN/dS ratio of the genes of interest, revealing whether the mutations were under purifying selection. The dN/dS ratio measures the rate of non-synonymous mutations over the rate of synonymous mutations, and we used this to determine how genes in strains evolved by seeing which mutations were favored. Using aligned coding sequences from wild *C. elegans* strains, we found that *drp-1*, *pdr-1*, and *atfs-1* were under purifying selection, suggesting that changes in these genes were often deleterious and, in turn, selected for through evolution. Conversely, *pink-1*, *eat-3*, and *fzo-1* each had a dN/dS ratio of 1, indicating that these genes are evolving randomly.

Changes in Protein Structure Due to Missense Variants:

Table 2 summarizes the Variant Effect Predictor (VEP) results for the six mitochondrial genes of interest, showing the

proportion of synonymous, missense, and stop-gained variants across CX11314 and EG4725. Table 3 further details the specific amino acid substitutions identified in each gene, highlighting the molecular changes that may affect protein structure and function.

Table 2: VEP results for candidate genes across CX11314 and EG4725. These variant distributions suggest gene-specific differences in evolutionary constraint.

Genes	Synonymous Variants	Missense Variants	Stop Gained
fzo-1	56%	44%	0
eat-3	52%	48%	0
drp-1	75%	25%	0
pink-1	59%	41%	0
atfs-1	55%	45%	0
pdr-1	53%	43%	3%

Table 3: List of specific amino acid substitutions found in the six genes of interest across CX11314 and EG4725 strains. Several substitutions involve changes in polarity or charge, which may alter protein stability and function.

Gene	Number of Variants	Specific Variants
fzo-1	5	1 P->S, 1 R->Q, 1 A->T, 1 V->M, 1 L->I
eat-3	5	1 I->M, 1 A->P, 1 K->R, 1 V->A, 1 A->T
drp-1	12	4 K->R, 4 L->I, 4 T->I
pdr-1	15	3 S->T, 4 E->K, 4 G->D, 2 L->R, 2 F->L
atfs-1	34	7 P->L, 7 Q->R, 7 S->T, 6 V->A, 7 Y->N
pink-1	5	1 E->K, 1 A->S, 1 P->S, 1 V->M, 1 N->D

The VEP and amino acid substitution analyses are summarized in Tables 2 and 3. VEP analysis of the six genes of interest in CX11314 and EG4725 revealed gene-specific synonymous, missense, and stop-gained variants that may influence protein structure or function. For *fzo-1*, 56% of variants were synonymous, and 44% were missense variants. Conservative substitutions included Valine to Methionine and Leucine to Isoleucine. Other substitutions, such as Proline to Serine, Alanine to Threonine, and Arginine to Glutamine, may alter polarity or charge and therefore could affect protein structure or function.

Secondly, the variations in *eat-3* consisted of 52% synonymous variation and 48% missense variation. In the case of *eat-3*, which consisted of 5 amino acid variations, most of the variations (Isoleucine to Methionine, Lysine to Arginine, and Valine to Alanine) were conservative. However, substitutions that change polarity, such as Threonine (polar) for Alanine (nonpolar), and changes in size, such as Alanine to Proline.

Even though *drp-1* had 12 missense variants, it had the highest synonymous variant percentages at 75%, suggesting that this gene has been evolutionarily conserved. In this case, *drp-1* had 4 of each type of missense variation: Lysine to Arginine, Leucine to Isoleucine, and Threonine to Isoleucine. All these, except Threonine to Isoleucine, are conservative changes that preserve its characteristic.

Unlike the other genes, *pdr-1* included 53% synonymous variants, 43% missense variants, and 3% stop-gained variants, suggesting that some variants may potentially lead to truncated protein products with altered functionality. Among the 15 missense substitutions, six substitutions, including Glycine to Aspartic acid and Leucine to Arginine, involved polarity or charge changes.

Interestingly, *atfs-1* had the highest number of missense variants at 34. 45% of the variants were missense, and 53% were synonymous; however, none of the substitutions caused by the missense variants resulted in a change in polarity. Instead, there were 7 substitutions (Glutamine to Arginine) that changed

from a neutral to basic pH; nonetheless, this will still result in an altered structure and function of the protein.

Finally, pink-1 showed 59% synonymous variants and 41% missense variants. Several missense substitutions may still be functionally important because they involve notable biochemical changes, including Glutamic acid to Lysine, Asparagine to Aspartic acid, Alanine to Serine, and Proline to Serine.

DEG Analysis Reveals Statistical Significance of Certain Transcripts:

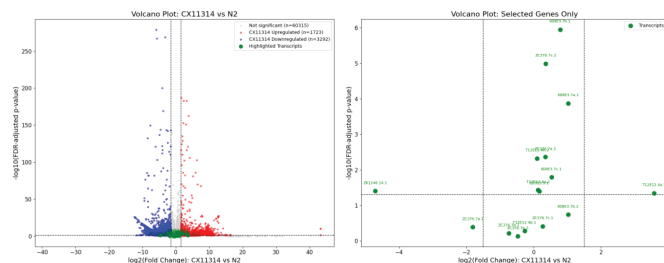


Figure 7: Volcano plots showing differential gene expression in selected mitochondrial genes of CX11314 and N2. Significant transcripts are highlighted based on emphasize mitochondrial genes adjusted p-values.

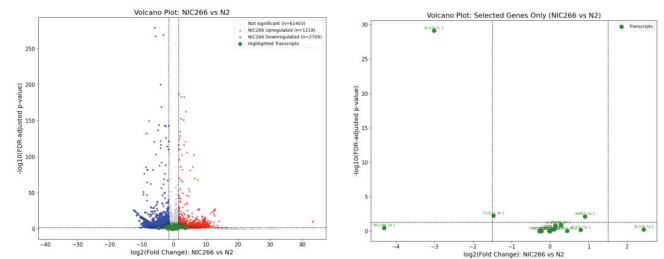
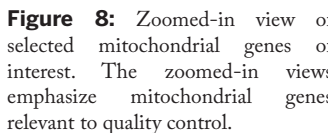
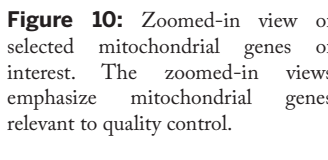


Figure 9: Volcano plots showing differential gene expression (log₂ fold selected mitochondrial genes of change vs. p-value) between NIC266 and N2. Significant transcripts emphasize mitochondrial genes are highlighted based on adjusted relevant to quality control. p-values.



The DEG results between CX11314 and N2 are shown in Figure 7, while Figure 8 provides a closer view of selected mitochondrial genes. The DEG results for NIC266 and N2 are shown in Figures 9 and 10. Volcano plots were used to visualize differential expression by comparing statistical significance (adjusted p-value) with the magnitude of expression change (log₂ fold change). Figure 7 shows that most transcripts corresponding to the genes of interest did not reach statistical significance in CX11314 compared with N2. However, the zoomed-in view in Figure 8 shows that several mitochondrial-related transcripts had observable expression shifts, including ZK1248.14.1 corresponding to *fzo-1* and T12E12.4a.1 corresponding to *drp-1*. Similarly, the NIC266 vs N2 comparison in Figures 9 and 10 showed that only a small subset of transcripts, including ZC276.7c.1 corresponding to *atfs-1*, T12E12.4b.1 corresponding to *drp-1*, and K083.7a.1 corresponding to *pdr-1*, reached statistical significance. Because NIC266 was used as a proxy for EG4725, these results should be interpreted cautiously and confirmed with direct EG4725 expression data in future work.

Higher Expression of *pdr-1*, *atfs-1*, and *eat-3*:

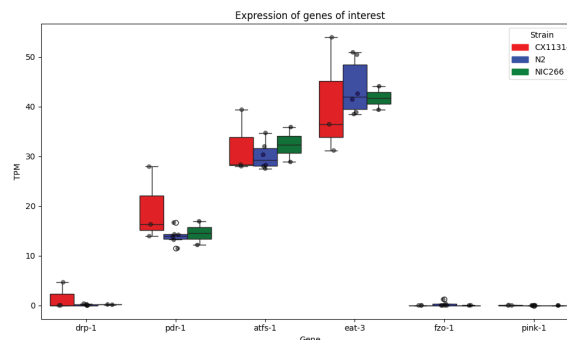


Figure 11: Box plot comparing TPM expression levels of selected mitochondrial genes across N2, CX11314, and NIC266. The figure highlights strain-specific expression patterns, including relatively high *pdr-1* expression in CX11314 and high overall *eat-3* expression.

The expression levels of the key genes across different strains are compared in Figure 11. The TPM values show that *eat-3* had the highest overall expression among the six genes, while *pdr-1* showed relatively higher expression in CX11314 than in the other displayed strains. In contrast, *fzo-1* and *pink-1* showed low expression across the strains. Although not all differences reached statistical significance, the strain-specific patterns suggest that expression variation may still contribute to fine-tuned mitochondrial regulation.

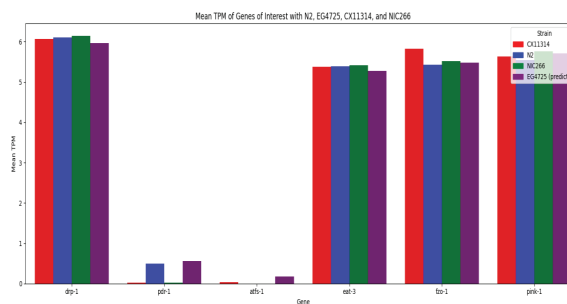


Figure 12: Bar graph comparing mean TPM values of N2, CX11314, NIC266, and predicted EG4725. The alignment between NIC266 and predicted EG4725 supports the use of machine-learning-based prediction as an exploratory approach when direct EG4725 RNA-seq data are unavailable.

The predicted gene expression profile for EG4725 is displayed in Figure 12. The model was trained on SNP and gene expression data from 204 *C. elegans* strains, and a neural network with LOOCV was used to estimate the missing EG4725 expression profile. NIC266 was excluded during validation because it was the genetically closest available proxy for EG4725. Spearman correlation analysis showed a strong prediction confidence ($\rho = 0.959$), supporting the reliability of the predicted profile for exploratory interpretation. However, because these values are predicted rather than directly measured, they should be confirmed through future RNA-seq experiments on EG4725.

Discussion

Overall, the combined analyses suggest that natural genetic variation in these six key genes may contribute to enhanced mitochondrial quality control in the wild *C. elegans* strains CX11314 and EG4725. Although many DEG results did not

reach statistical significance, the observed expression patterns, PCA clustering, phylogenetic divergence, and VEP results together support the possibility that these genes fine-tune mitochondrial regulation under different genetic backgrounds. The missense variants identified in Tables 2 and 3 may alter protein structure or function through changes in polarity, charge, pH, or molecular size. In addition, the predicted EG4725 expression profile showed higher expression of *pdr-1* and *atfs-1*, suggesting a potential link with stronger mitochondrial quality control. These findings indicate that naturally occurring variation in CX11314 and EG4725 may contribute to fitness under environmental pressure and provide candidate targets for future mitochondrial disease research.

Therefore, we propose using CRISPR-Cas9-mediated homology-directed repair (HDR) to swap both promoter and protein-coding regions of selected genes of interest. This method is appropriate because CRISPR-Cas9 genome editing allows precise replacement of genomic regions with donor DNA sequences.²⁵ Specifically, we propose replacing the N2 promoter and coding regions of *pdr-1* and *atfs-1* with the corresponding regions from EG4725. This proposal is based on the predicted higher expression of *pdr-1* and *atfs-1* in EG4725, as shown in Figure 12. Because Figure 12 presents expression on a logarithmic scale, even visually modest differences may represent larger underlying expression differences. These experiments would help determine whether the EG4725 alleles directly contribute to improved mitochondrial quality control.

To appropriately conduct these experiments, we propose using four experimental groups: N2 (control), N2 carrying the EG4725 *pdr-1* allele, N2 carrying the EG4725 *atfs-1* allele, and EG4725 (baseline). This design would allow the functional effects of individual EG4725 variants to be tested within the N2 background and compared with the native EG4725 strain. It would also reveal whether specific alleles are sufficient to change mitochondrial phenotypes or behavior. Phenotypic and behavioral assays could assess mitochondrial morphology, mitophagy-related activity, ATP and ROS production, stress resistance, lifespan, and overall activity. These experiments would help clarify how genetic variation drives phenotypic differences and may provide a foundation for future therapeutic approaches targeting mitochondrial dysfunction.

From a practical perspective, this study provides a candidate-gene framework that can guide future experimental validation rather than functioning as a final causal proof. The results help prioritize *pdr-1*, *atfs-1*, and related mitochondrial quality-control genes for follow-up testing in *C. elegans*. If validated experimentally, these naturally occurring variants may help explain how organisms maintain mitochondrial homeostasis and may inform broader studies of aging, stress tolerance, and mitochondrial disease mechanisms.

■ Conclusion

This study investigated how natural genetic variation in mitochondrial health-regulating genes contributes to mitochondrial resilience in the *Caenorhabditis elegans* strains CX11314 and EG4725. By integrating variant analysis, gene

expression information, and functional interpretation of key mitochondrial pathways, we identified consistent differences between these strains and the reference N2 strain. The results suggest that naturally occurring missense variants and regulatory differences in genes involved in mitochondrial dynamics, mitophagy, and stress response may collectively enhance mitochondrial stability and function. Our findings highlight the importance of studying natural genetic diversity, rather than relying solely on laboratory-induced mutations, and how mitochondrial health is maintained under physiological conditions. The observed associations between genetic variation and improved mitochondrial resilience provide a useful framework for exploring how similar mechanisms may operate in other organisms. This work also demonstrates how combining genetic data with pathway-level interpretation can generate biologically meaningful insights, even in relatively small datasets. While the present study focuses on *C. elegans*, the analytical approach may be extended to other model systems and complex traits related to cellular health and aging. Future studies could further validate these findings through targeted functional experiments and explore the broader implications of natural mitochondrial variation in stress tolerance and disease susceptibility.

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■ Author Contribution Statement

Aaryan S., Mansi M., and Mohammed M. contributed equally to the writing of the paper, data collection, programming, graph and table generation, and data analysis. Generative AI tools, such as ChatGPT, were used to assist with Python programming, while Grammarly was employed to improve writing clarity and grammar.

The Cost of Late Diagnosis: A Synthetic-Data-Based Study of Lung Cancer Survival by Stage

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ABSTRACT: The most common cause of cancer mortality in Korea is lung cancer, and patients are usually diagnosed at a late stage. This article examines the association between the diagnostic stage and survival based on a large population representative synthetic electronic health record dataset. A stage-based analysis of survival was performed, followed by an analysis of postoperative outcomes and sex-specific trends of survival. A considerable loss in survival was found between the early (Stages I-II) and locally advanced (Stage III) stages. The differences in survival between Stages III and IV were insignificant, indicating that the inability to resect surgically is a clinically relevant prognostic marker. Sex-stratified tests found the following differences in survival patterns: Women had steeper declines at certain stages, whereas men had a gradual decline. Multivariate analysis suggests that sex is an independent predictor of survival. These results highlight the role of early diagnosis and indicate that sex may play a role in stage-specific survival. The use of synthetic data obviates privacy issues, but it must be validated by real-world clinical data. Finally, this study, in addition to justifying the importance of early diagnosis, indicates areas for future research to improve the countrywide screening and management of lung cancer.

KEYWORDS: Medicine, Epidemiology, Lung Cancer, Stage-Specific Survival, Korean Population.

■ Introduction

Lung cancer is the leading cause of cancer-related mortality worldwide, accounting for close to one-fifth of cancer-related deaths across the world.¹ This is a major global public health concern, especially in high-income nations, where its incidence and mortality have been rising in the last few decades. The World Health Organization (WHO, 2025) indicates that the incidence of cancer has increased worldwide, with the number of deaths caused by lung cancer ranking first. Lung cancer is the major cause of cancer-related deaths in South Korea, with an age-standardized rate of incidence of approximately 595.4 in 100,000 men and 543.5 in 100,000 women in 2024.² The crude mortality rate of lung cancer is high at 23.0 per 100,000, even with progress in the medical sphere.³ The main cause of such high mortality is that lung cancer is frequently only diagnosed at its advanced stages, i.e., in Stages III and IV, which significantly lowers the treatment possibilities and survival rates.⁴

Most cases are either locally advanced or distant metastasis.⁵ Unlike other types of cancer, which are usually identified early because of particular symptoms or routine screening, lung cancer, in most cases, is not diagnosed until it has spread outside the local region. This leaves limited treatment options and low chances of survival, as the survival rates show a marked decline in survival as cancer advances: The five-year survival rate of non-small cell lung cancer (NSCLC) stands at about 82%, which is Stage I, but is only about 10% in Stage IV.² This substantial difference in survival underlines the need to detect lung cancer early enough and hence the need for stronger screening tools to detect the disease, especially among high-risk groups, particularly in South Korea, where the incidence is high and

early detection measures are needed to reduce the increasing burden of lung cancer.

Cancer screening programs are important in the detection of cancers at earlier and more treatable stages. The benefits of early detection have already become apparent in the case of other types of cancer, especially cervical, colorectal, and breast cancer. In the United States, cervical cancer rates have declined by about 55 per cent in the last few decades, and a European study found that extensive cervical cancer screening programs reduced mortality rates by 41-9%.^{6,7} Likewise, a vast Swedish cohort study showed that the death rate of women with breast cancer has declined among the regularly screened population.⁸ These screening programs have already shown the possibility of identifying cancer during its early stages, allowing interventions to be provided earlier, leading to increased survival rates and reduced costs of treatment in the long term.

The same success has not been achieved in screening programs for lung cancer as for cervical, colorectal, and breast cancers; however, despite its high mortality rate, especially at the advanced stages, which, as noted, is the leading cause of cancer-related death among all cancers.⁴ Although there is an extensive body of research revealing the benefits of early screening of other types of cancers, the data on survival rates of lung cancer by stage of diagnosis remain limited, especially in Korea. Most research has investigated general cancer occurrence or death without considering the important effect of the diagnostic stage on patient outcomes, leaving us with a lack of information on the stage-specific survival outcomes of lung cancer patients in Korea, particularly in the earlier stages,⁹ when the cancer is localized and may be removed via surgical resection or alternative curative interventions.⁵

Staging lung cancer is an important clinical process in which the Tumor Node Metastasis (TNM) classification is employed to determine the progression and prognosis of lung cancer. Stage I and II lung cancer are usually localized and have high survival rates after surgical procedures, but Stage III and IV cancer are more widespread and often require a combination of therapies, including chemotherapy and radiation; the curative treatment options are limited because lung cancer at these stages is typically surgically inoperable, and survival rates are much reduced.⁵ More efficient tools for the early detection of lung cancer are thus needed to facilitate diagnosis at the earliest possible stage.

A significant issue in large-population-based research on lung cancer survival has been difficulties in gaining access to extensive patient-level data while protecting patient privacy. Synthetic electronic health record (EHR) data offers one solution to this dilemma. EHR data are generated from de-identified real-world data, which protects patient privacy but still offers a comprehensive picture of patient outcomes. These data can be useful for examining survival trends and other significant health outcomes of large groups of people, particularly in nations like South Korea, where conventional data access may be limited by privacy issues.¹⁰ This study utilized a large synthetic EHR data set to examine the relationship between the diagnostic stage and survival of Korean lung cancer patients. The analysis of this data helps mitigate the limitations of traditional registries, which do not tend to give extensive stage-specific survival data, and allows the results to be applied to the broader population of Korea. Accordingly, this study aimed to address a gap in our knowledge of the stage-specific survival rates of Korean lung cancer patients.

In particular, this study examines the survival times by stage at diagnosis in patients with lung cancer in Korea. We hypothesize that the later the patients are diagnosed, the shorter their survival times, as the stage at diagnosis is anticipated to be a key predictor of survival.¹¹ In addition, this study examines the possibility of a moderating effect of sex on survival results, as sex-related disparities in lung cancer survival have been reported, such that women generally have a steeper survival curve at certain stages.⁴ The results of this study offer insight into the stage-specific survival of lung cancer patients in Korea and their potential implications for health policy to enhance early detection and treatment measures, as well as for the design and evaluation of national screening programs like the Korean Lung Cancer Screening Program (NLCSP), which has demonstrated the effectiveness of early detection, but without full knowledge of the overall survival outcomes.¹²

■ Methods

This retrospective, observational study used synthetic electronic health record (EHR) data that the South Korean government synthetically created from the country's national cancer registry and tertiary hospital records using artificial intelligence (AI)-driven modeling. In short, the dataset was built from synthesized real-world clinical information that represents the demographic and clinical characteristics of

Korean cancer patients but does not contain any identifiable patient information. Using CONNECT, a large healthcare data platform supported by the National Information Society Agency (NIA). The CONNECT platform includes structured clinical data from the colorectal cancer clinical library (converted to lung cancer records) and the national cancer registry (supplemented with tertiary hospital data) that covers most cancer patients in Korea to ensure representativeness at the population level.

The primary outcome was the survival time in months from the date of original diagnosis of lung cancer to the date of death. No censoring was performed because all the subjects in the data were deceased. Stage diagnosis was the primary independent variable that was determined based on Tumor-Node-Metastasis (TNM) guidelines, where T indicates the size and extent of the primary tumor; N indicates the amount of a regional lymph node that is involved; and M indicates the occurrence of distant metastasis. Based on these criteria, a stage (I–IV) was given to every patient. To test the study hypothesis, the four standard stages were categorized as early stage (Stages I–II) or advanced stage (Stages III–IV). There was a list of additional variables that could be used as covariates, not all of which were in the key model: demographic variables, including age, sex, previous treatment history (surgery, chemotherapy, and radiation); comorbid diseases like chronic obstructive pulmonary disease (COPD) and pulmonary fibrosis; and other pertinent clinical outcomes.

R (version 4.5.1) was used for all statistical analyses. Continuous variables were described with means (M) and standard deviations (SD), whereas categorical variables were described in the form of frequencies (n) and percentages (%). Linear regression (OLS) was conducted where the outcome was the survival days (OVRL_SRVL_DTRN_DCNT) and stage at diagnosis (I–IV) was the primary independent variable. OLS was used since all individuals were deceased; hence, the survival period was known for all cases. Although the use of OLS regression in this case might be appropriate, the data are only for deceased subjects, which can limit its representativeness through selection bias by excluding longer-lived subjects, resulting in underestimated survival times and thereby biased stage comparisons. Therefore, the findings reflect only deceased subjects. In this context, OLS regression provides a straightforward approach to estimating mean differences in survival time across groups, as complete survival durations are observed for all individuals. However, this approach differs from conventional survival analysis methods, such as Kaplan–Meier estimation or Cox proportional hazards models, which are typically used when censoring is present. Therefore, the findings should be interpreted with caution, as the analytic framework and sample structure might have influenced the observed associations. Sex (BSPT_SEX_CD) was incorporated as a covariate in the adjusted model, and further models were stratified by sex (male and female). The stage x cancer subtype (BSPT_FRST_DIAG_CD) interaction model was fitted to determine whether the differences in survival based on stage were different across subtypes. A t-test was used to compare the survival between stages (e.g., Stage I vs II/III/IV) and oth-

er outcomes (e.g., days from first operation to death, days from first diagnosis to death) across stages, with the results visualized as boxplots, violin plots, and heatmaps. Model diagnostics were conducted through the analysis of the residual distributions in terms of the Q-Q plot of the linear regression models. Two-sided tests were used to determine statistical significance, with $p < 0.05$, and 95% confidence intervals were presented; no multiple testing correction was applied to exploratory t -tests.

As this study used entirely synthesized EHR data without recognizable patient data, patient confidentiality was maintained, and institutional review board (IRB) approval and informed consent were not needed. The study complied with the ethics of researching human health records by using synthetic data that is publicly available.

■ Results and Discussion

We now turn to the findings of our stage-specific survival analyses. The demographic and clinical characteristics of the study population are provided first, followed by the comparison of the survival outcomes by diagnostic stage. A total of 4,307 lung cancer patients were included in the study (Table 1). The mean age at diagnosis was 66.2 years ($SD = 8.8$). Most patients were male (87.9%, $n = 3789$), and only a minority were female (12.0%, $n = 518$), which is in agreement with the fact that Korean men have higher smoking rates and consequently higher lung cancer morbidity rates than Korean women. In terms of stage at diagnosis, most patients presented with advanced-stage disease: Only 19.3% ($n = 832$) of patients presented with Stage I disease, while 41.2% ($n = 1,773$), 22.5% ($n = 969$), and 16.5% ($n = 710$) presented at Stages II, III, and IV, respectively. This indicates that most patients in this cohort were not discovered by early detection and screening, highlighting the need for better lung cancer screening and diagnostic methods.

Treatment type was also influenced by stage. Surgery was most common among early-stage patients, in which curative resection was possible for Stages I–II disease. The small difference in survival between Stages III and IV is notable. Although it may reflect the loss of the possibility for surgical resection at the advanced stage, other factors must be taken into account. They include the heterogeneity of therapeutic strategies, as patients at later stages might receive different forms of treatment such as chemotherapy, radiation therapy, or any other type of treatment again, reflecting the utility and applicability of these treatments in the absence of surgical options. As such, the distribution of stage at diagnosis in this cohort is in line with the natural history of lung cancer and is a useful context for the subsequent survival data.

Table 1: Baseline characteristics of lung cancer patients ($N = 4,307$). The study group consisted predominantly of male patients diagnosed with advanced-stage disease, with more than 80% presenting at Stages II–IV. This pattern indicates a low likelihood of early disease detection in this population.

Variable	Category	Frequency (n)	Proportion (%)	Mean	SD
Sex	Male	3789	87.97		
	Female	518	12.03		
Age at diagnosis	-	-		66.22	8.84
Diagnostic stage	I	832	19.32		
	II	1773	41.17		
	III	969	22.5		
	IV	710	16.48		

Survival by Stage:

Survival time decreased as the stage at diagnosis increased (Figure 1). Patients with Stage I disease had the longest survival (mean = 886.1 days; median = 837.5 days). And those with Stage II disease have a slightly shorter survival (mean = 831.8 days; median = 761.0 days). The decline in survival was steeper for patients with Stage III disease (mean = 747.7 days; median = 652.0 days). Unexpectedly, however, the mean survival for Stage IV patients was only marginally longer (mean = 754.0 days; median = 689.5 days) than for Stage III patients, although this difference was not significant. There is a non-linear relationship between stage and survival, and this may be a result of heterogeneity in treatment assignment across patients with advanced disease, differences in patient biology, or palliative effects.

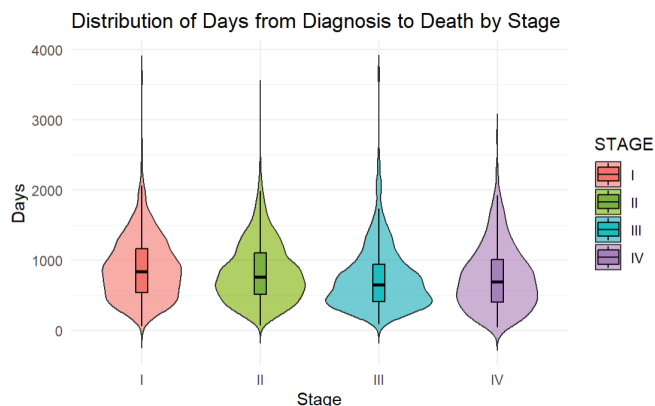


Figure 1: Survival duration (days) from diagnosis to death by diagnostic stage in Korean lung cancer patients. There is a marked difference in survival between Stage I–II disease and Stage III disease, with only a slight difference between Stages III and IV. This trend suggests that the loss of surgical resectability, rather than further disease progression, is a major determinant of survival.

Pairwise comparisons clarified the foregoing stepwise trend (Figure 2). Compared with Stage I disease, median survival decreased significantly at all later stages, by 54.4 days (Stage II, $p < 0.01$), 138.4 days (Stage III, $p < 0.01$), and 132.1 days (Stage IV, $p < 0.01$). Similarly, Stage II patients had longer survival than those with Stage III or IV disease by 84.0 days ($p < 0.01$) and 77.7 days ($p < 0.01$), respectively. By comparison, the difference in median survival between Stages III and IV was minimal (6.3 days, $p = 0.782$), suggesting that once the disease

is no longer resectable, further progression has little impact. These findings highlight the importance of early detection for survival.

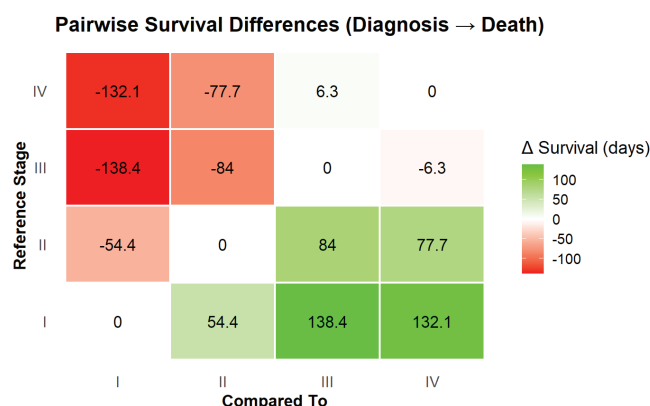


Figure 2: Heat map - diagnosis-death. Survival differences are pronounced when comparing Stage I with Stage IV disease, but are minimal between Stages III and IV. This pattern supports the interpretation that the transition from surgical resectability to non-resectability, rather than further stage progression, is a key determinant of survival.

Post-Operative:

Survival Within the subgroup of patients who were resected, survival progressively decreased at more advanced stages (Figure 3). The average survival days after surgeries were 844.7 (median = 805.5) in Stage I, 795.8 (median = 724.0) in Stage II, 725.3 (median = 627.0) in Stage III, and 711 (median = 645.0) in Stage IV. Regression analysis indicated that surgery performed at earlier stages was significantly associated with greater survival benefits. These results are summarized in the triangular heatmap (Figure 4): Survival declined markedly from Stages I–II to Stages III–IV. Collectively, these data highlight the need for curative resection before local or systemic metastasis and indicate that the surgical prognosis is associated with poorer outcomes once the disease is no longer resectable.

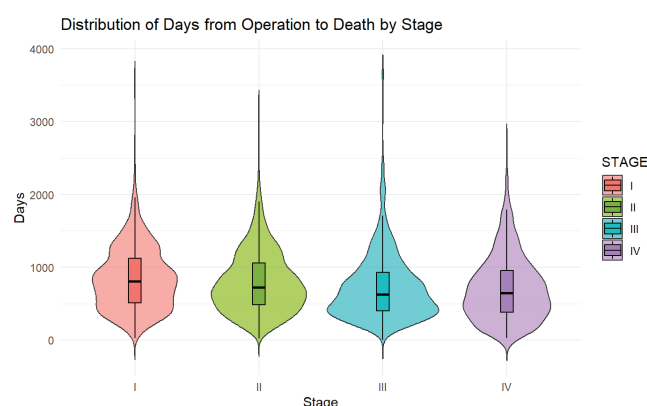


Figure 3: Post-operative survival duration (days) from operation to death by diagnostic stage. Post-operative survival decreases progressively with advancing stage at diagnosis, with the greatest survival observed in patients who undergo surgery at early stages. These findings suggest that the survival benefit of surgery diminishes once tumor burden exceeds resectable limits.

Pairwise Survival Differences

Operation → Death (by Stage)

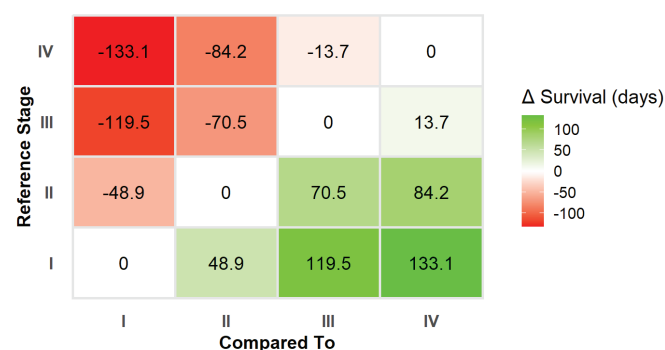


Figure 4: Heat map - operation-death. Large survival advantages are observed for patients undergoing surgery at earlier stages, whereas post-operative survival differences between Stages III and IV are minimal. This pattern indicates that the survival benefit of surgery declines once the disease exceeds resectable limits.

Sex Effects on Survival:

Sex-stratified analysis showed differences in stage-specific survival (Tables 2 and 3), such that survival decreased sharply in women at stages II (-179.8 days, $p < 0.001$) and again at IV (-327.0 days, $p < 0.001$) compared to stage I, with a non-significant decline at III (-181.8 days; $p = 0.165$). These findings suggest that female patients may lose their survival benefit more rapidly during the first phase, possibly at least in part due to underlying biological differences, tumor behavior, or response to therapy.

Male patients demonstrated a more stepwise and regular decrease: Survival was reduced by -26.9 days at Stage II ($p = 0.178$), -84.4 days at Stage III ($p < 0.001$), and -102.6 days at Stage IV ($p < 0.001$). This more stable trend is thought to be due to the greater underlying disease burden because of longer periods of smoking-induced pathology among Korean men.

The model was further adjusted for sex and cancer subtype to examine the interactions between these patient characteristics. Although there was a trend of decreasing survival with increasing stage, the effect of cancer stage did not reach statistical significance when adjusted for other prognostic factors for the disease. Compared to Stage I, Stage II survival was lower by -222.9 days ($p = 0.343$), by -263.8 days for Stage III ($p = 0.168$), and by -112.0 days for Stage IV ($p = 0.402$). Unlike stage, sex was also an independent predictor for survival in the multivariate analysis: Men had a predicted survival 116 days shorter than women did on average ($p < 0.001$). Between cancer subtypes, minimal nonsignificant differences were found ($p > 0.1$ for all).

These results suggest that sex is a significant effect modifier for stage-specific survival in Korean lung cancer patients. For women at specific stages of disease, survival declines more steeply; for men, it is a more gradual decline. The sex difference in survival did not vary by cancer subtype and may need to be considered in lung cancer screening, staging, and treatment.

Table 2: Sex-stratified regression of survival duration by stage at diagnosis in Korean lung cancer patients. Stage-specific survival patterns differ by sex, with women exhibiting sharper declines in survival at selected stages, while men show a more gradual reduction with advancing stage. These findings indicate that sex modifies the relationship between diagnostic stage and survival.

Stage	Female		Male	
	Survival Deviation from Stage 1	p-value	Survival Deviation from Stage 1	p-value
II	-179.8	<0.001	-26.9	0.178
III	-181.8	0.165	-84.4	<0.001
IV	-327	<0.001	-102.6	<0.001

Table 3: Sex-adjusted regression of survival duration controlling for cancer subtype. After adjustment for diagnostic stage and cancer subtype, sex remains an independent predictor of survival, whereas stage effects are reduced. This suggests that sex-related factors contribute to survival differences beyond disease stage alone. Diagnosis codes correspond to ICD-10 classifications for lung cancer (C34.0–C34.9).

Variable	Estimate	P-value	97.5 % Confidence Interval (Lower–Upper)
Stage II	-222.86	0.343	-461.06 to +15.34
Stage III	-263.79	0.168	-374.88 to -152.70
Stage IV	-111.97	0.402	-261.79 to +37.85
Sex (female: control)	-116.04	<0.001	-191.44 to -40.64
ICD-10 C34.1	7.21	0.94	-189.38 to +203.80
ICD-10 C34.2	-158.46	0.122	-200.59 to -116.33
ICD-10 C34.3	-76.34	0.43	-189.78 to +37.10
ICD-10 C34.8	52.37	0.821	-452.53 to +557.27
ICD-10 C34.9	-21.53	0.825	-191.07 to +148.01

* ICD-10 C34.1: Malignant neoplasm of the upper lobe, bronchus, or lung
* ICD-10 C34.2: Malignant neoplasm of the middle lobe, bronchus, or lung
* ICD-10 C34.3: Malignant neoplasm of the lower lobe, bronchus, or lung
* ICD-10 C34.8: Overlapping lesion of the bronchus and lung
* ICD-10 C34.9: Malignant neoplasm of the unspecified part of the bronchus or lung

In this nationwide, large Korean cohort of 4,307 lung cancer patients, the majority of whom were male, most patients were diagnosed at an advanced stage, consistent with limited early detection. Survival was reduced from stage to stage with a marked decline between early-stage (I–II) and locally advanced-stage (III) and a nearly flat slope between stages III and IV. There was a significant survival advantage in patients who underwent surgery at an early stage, highlighting the importance of early diagnosis and management. There were differences by sex in stage-specific lung cancer survival, however. Women had a steeper decline in survival at some stages,

while a less pronounced decrease was found among men, and sex was an independent predictor of survival adjusted for stage and subtype, with males living an average of 116 days shorter than females ($p < 0.001$). These findings suggest that stage at diagnosis and sex were key factors associated with overall survival among Korean patients with lung cancer.

This study sets out to test the association between stage at diagnosis and survival time among Korean lung cancer patients based on a large-scale synthetic electronic health record (EHR) dataset. The results provide insights into the stage-specific survival outcome of lung cancer in Korea, the significance of early diagnosis, and the critical role of the stage at diagnosis in terms of long-term survival. Also, the research highlights the impact of sex on survival rates, suggesting that sex-specific treatment could help improve lung cancer survival.

Effect of Stage of Diagnosis on Survival:

The findings of this study are in line with those of previous studies, which indicated that the duration of survival declines substantially as the diagnosis stage progresses.⁹ Patients in Stage I had the highest survival times, with a mean survival time of 886.1 days. This result highlights the prognostic benefit of early-stage diagnosis and is consistent with the worldwide knowledge of the prognostic value of cancer at an early stage that is localized and can be treated with surgery, giving the highest possibility of survival.¹¹ Survival of Stage II patients was relatively good; however, there was a marked decrease from that of Stage I, and a mean survival of 831.8 days was observed. Nevertheless, the strongest outcome was observed when the comparison was made between Stages III and IV. Although it may be reasonable to assume that there would be a greater difference in outcomes between these stages, the results showed that there was minimal difference in survival of patients in these two stages, with a mean survival time of about 754.0 days in Stage IV and 747.7 days in Stage III. This implies that when the disease is at Stage III, additional development to Stage IV was not associated with a substantial difference in the chances of survival. This aligns with other research results that show that when the disease has reached local progression and is no longer controllable, the prognoses do not improve with further progression of the disease.⁴

Sex Effects on Stage-Specific Survival:

One of the notable findings of this study was the sex-specific differences in the survival trends. The survival of women declined markedly in Stage II, and again in Stage IV, but there was a slower decline in men in the stages. The findings are consistent with those of the earlier findings of better survival outcomes in women at various stages of lung cancer, potentially because of biological differences, including hormonal or tumor biological variations.¹³ Conversely, the decline in survival was more linear among men, who smoke more and, therefore, have a greater chance of encountering lung cancer.⁴ The identified sex differences in survival results indicate that sex-specific approaches to lung cancer diagnosis, therapy, and screening are necessary. Multivariate analysis showed that sex remained an independent predictor of survival, where men lived an average

of 116 days shorter than women, despite the cancer stage and subtype. The results can be used to support the hypothesis that biological and behavioral disparities between men and women influence survival outcomes and that sex-specific interventions can help to achieve better outcomes in terms of survival.¹³ These findings are important in informing the optimal public health policies, which might help in establishing more individualized policies on lung cancer detection and treatment.

The Rationale of Early Detection and National Screening Programs:

The results in this research indicate that earlier diagnosis is important in helping lung cancer patients have a better chance of survival. As Stage I patients were found to be associated with substantially higher survival rates, there is an indication that early diagnosis is an important factor in prolonging the life span. The information also indicates that the difference between Stage I and Stage III is large, whereas the difference between Stage III and Stage IV is comparatively minor. This shows that the greatest survival advantages are achieved when the disease is detected before reaching a stage where curative treatment is no longer feasible. This finding offers support in favor of advancing the lung cancer screening programs, including the Korean Lung Cancer Screening Program (NLCSP), which has suggested the potential to identify early-stage cancers. The effectiveness of screening programs for other types of cancer, like cervical and colorectal cancer, further supports the need to implement cancer screening programs on a population level.⁷ There are examples of programs that focus on early detection of cancer that have been credited with greatly lowering mortality of people in several countries, and a similar program targeting lung cancer may contribute to reductions in mortality.⁶ Although the initial outcomes of the Korean Lung Cancer Screening Project (K-LUCAS) are promising, the results require confirmation using the survival data by stage to optimize these programs for national implementation.¹⁴

Limitations of Synthetic EHR Data:

Although there are several benefits to the synthetic EHR data set utilized in this research, especially regarding privacy issues and the delivery of population-wide information, it has several limitations that should be borne in mind. Although synthetic data are helpful in a large-scale epidemiological study, they might be inadequate in reflecting the heterogeneity of clinical data in the real world, especially in terms of treatment procedures and patient behavior. As an example, the data employed in the present study did not include such variables as smoking history, socioeconomic status, and comorbidities, which have also been reported to influence lung cancer survival.¹⁵ In addition, the synthetic dataset does not have the same clinical detail as real-world data, so it is not generalizable to actual populations. However, synthetic data can provide information about stage-specialized survival with greater accessibility and replicability. Another limitation is that the synthetic EHR data do not provide detailed treatment data. The choice of treatment modalities, i.e., chemotherapy, surgery, and radiation, is also a key determinant of survival outcome among patients with lung

cancer.¹⁶ Further research based on actual clinical information must incorporate treatment-related variables for a better picture of the factors underlying the difference in survival rates between stages. Also, despite the differences in survival rate between men and women, there remains an obvious bias in the population, with more men (88%) than women (12%). As a result, the validity of sex-based survival difference comparisons might be questionable because of the large difference in the number of patients by sex, which can pose problems for statistical analysis. The specific mechanisms involved were also not explored in this study. Thus, any explanations that attribute these effects to hormone levels or biological processes can be considered hypothetical. A final limitation of the methodology was the use of synthetic data derived by converting records of colorectal cancer to records of lung cancer. Although this approach facilitates research in the absence of actual data on lung cancer, the resulting data may not represent the specific biological and clinical features of patients with lung cancer. This implies that some of the findings of the current method may not be entirely reflective of actual clinical settings.

Future Research:

Directions The present research contributes to research on lung cancer survival, yet substantial gaps remain. Future research should aim to confirm these results with real-world clinical data, which would overcome the limitations of synthetic data and create a more accurate image of the outcomes of lung cancer survival. Specifically, this will be necessary to determine how other factors, including treatment modalities and comorbidities, can affect survival, as such factors are likely to interact with stage at diagnosis and sex to influence results. Also, further research is needed to clarify the mechanisms underlying the sex-specific difference in survival found in this study. Knowledge of the biological, hormonal, and behavioral mechanisms that underlie these differences may inform the development of more targeted treatment strategies and enhance survival outcomes.

■ Conclusion

A population-representative synthetic electronic health record dataset was used in this study to explore the impact of stage diagnosis on the prognosis of Korean lung cancer patients. The survival in the cohort declined between early (Stages I, II) and locally advanced disease (Stage III), with little difference observed between Stages III and IV. The minimal differences in the results after the disease passes the threshold of surgical resectability are an indication that this pattern may reflect the impact of delayed diagnosis, once curative surgical options may no longer be feasible. In practice, the benefits of survival may be expected not in cases of aggressive treatment of the disease at a very advanced stage, but in the diagnosis of the disease at an earlier stage, so that more patients may receive treatment while surgical resection remains feasible.

Post-operative analyses supported these findings. In patients with surgery, survival was decreased with advancing stages, and in those who had surgery in Stages I–II, clear differences in

survival were observed from those who received surgery after a delay in Stages III or IV. These findings highlight the importance of diagnosis at an early stage and timely curative resection and lend credence to the argument for enhancing national responses that shift patients left on the diagnostic timeline through improved assessment of risk, earlier referrals, and organized screening of high-risk populations.

Sex-stratified models indicate that stage does not work in isolation. Thus, sex is associated with differences in outcomes, as the abrupt loss of survival in women occurred at certain stages, whereas men experienced a more gradual loss. After adjusting for stage and cancer subtype, sex was an independent predictor of survival, such that men had shorter survival times on average than women. This trend suggests biological, hormonal, behavioral, or treatment-based differences between men and women that can alter how the stage is translated into survival time. This implies that generalized screening and management strategies might overlook the distinct sex-specific risks and intervention opportunities, which can affect the general health of the population and the health care delivered to its members.

The availability of computer-generated EHR data enabled this study to provide detailed, stage-specific survival estimates on a national scale that do not place patient privacy at risk. At the same time, however, synthetic data cannot be entirely substituted for real clinical data. Smoking history, socioeconomic status, comorbidity burden, and detailed treatment pathways are important elements that are not fully represented in the dataset, and the absolute figures generated by the present study must be viewed with some reservations. Future research is needed to validate these findings with data from real clinical registries and incorporate more variables in the analysis to enhance our understanding of the interactions of stage, sex, treatment, and background risk in practice.

Overall, this study suggests that stage at diagnosis and sex are both key factors of survival in Korean lung cancer patients and that the survival declines most markedly after the disease has reached the later stages, when it cannot be surgically removed. It gives quantitative support to the case for earlier detection, more decisive application of curative surgery while the disease remains resectable, and more subtle sex-specific screening and management approaches. Simultaneously, it identifies unresolved issues: the necessity to test synthetic-data results, the necessity to incorporate behavioral and social risk factors into the survival models more thoroughly, and the necessity to refine how national lung cancer screening programs can be fine-tuned to detect more cancers before the disease progresses beyond treatable stages.

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I am a Year 12 student studying mathematics, biology, and chemistry, and I aspire to study medicine at university. I have strong motivation for this field due to my ambition to combine clinical practice with research to improve diagnosis, treatment, and health policy.

Ecosystem Rehabilitation Approach Using Oriental Herbs to Detour Agricultural Antibiotic Resistance from Antibiotic Overuse

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ABSTRACT: This study reconsiders traditional plant extracts as sustainable alternatives to synthetic antimicrobials in response to ecological concerns and rising resistance in *Agrobacterium tumefaciens* (*A. tumefaciens*), the agent of crown gall. Aqueous extracts of garlic, ginger, cinnamon, and star anise (prepared from 75 g plant material each) were evaluated by Kirby–Bauer disk diffusion across a concentration series using isolates from crown galls. Garlic and ginger produced the largest inhibition zones and were bactericidal at 25% concentration; star anise was bactericidal at 25% with smaller zones, while cinnamon required 50% to show bactericidal effects. In radish assays, all treated seeds germinated, and garlic and ginger—though most effective against bacteria—reduced early seedling vigor at their bactericidal concentrations compared with water controls. These results partially support the hypothesis that herbal extracts can suppress *A. tumefaciens* without preventing germination, but they also reveal potential phytotoxicity at concentrations needed for bacterial control. The study highlights the dual outcomes of antimicrobial efficacy and growth inhibition and recommends optimization of concentration and formulation to maximize crop protection while safeguarding plant growth before field application.

KEYWORDS: Microbiology, Antimicrobials and Antibiotics, Natural Herb Extracts, *Agrobacterium tumefaciens*, Antimicrobial Resistance.

■ Introduction

The increasing use of synthetic antimicrobials in agriculture and medicine has raised environmental concerns and contributed to the global spread of antibiotic resistance.^{1,2} Plant-derived compounds are thus being revisited as eco-friendly alternatives for antimicrobial control.^{2,3} In Korean fermented foods like kimchi, lactic acid bacteria play crucial roles in fermentation, preservation, and health-related properties of the final product.^{4,5}

Recent studies show that garlic and ginger extracts can inhibit multidrug-resistant bacteria, while cinnamon- and star anise-based essential oils also suppress bacterial growth and biofilm formation.^{1,6} Additionally, research on *Agrobacterium tumefaciens* has demonstrated antimicrobial activity from plant essential oils, indicating this pathogen as a suitable model for testing natural antimicrobials.⁷

This study investigates the antimicrobial effects of garlic, ginger, cinnamon, and star anise extracts against *Agrobacterium tumefaciens*.⁷ Unlike previous studies focusing mainly on single plant materials or different bacterial targets, this work compares multiple traditional ingredients under identical experimental conditions to evaluate their potential as sustainable alternatives to synthetic antimicrobials.^{1,6,7}

■ Methods

LB Agar Preparation:

Figure 1 shows the LB Agar preparation. (1) Double-distilled water (DDH₂O) was prepared. (2) 25 g of LB powder was mixed with DDH₂O. (3) 16 g of agar powder was mixed

with the solution. (4) The mixed LB agar has been autoclaved at 121 °C for 20 minutes for sterilization. After the autoclave, the LB agar solution was stored at 25 °C. (5) The solidified LB agar was melted by repeated short microwaves and poured into sterilized Petri dishes (about 10 mL per plate). After solidifying LB agar, the Petri dishes were stored at 4 °C for the first two days before the following experiments.

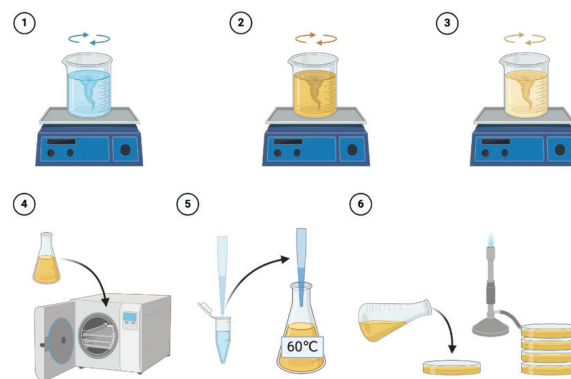


Figure 1: LB Agar preparation. This figure documents the sterile LB agar preparation protocol used to culture *Agrobacterium*; plates prepared as shown supported subsequent colony growth used throughout the study.

Agrobacterium Collection:

As Figure 2 illustrates, (1) Crown gall was collected from an oak tree in Kowloon City, Hong Kong, by a chopping knife. (2) After soaking them in DDH₂O for 24 hours, the crown gall was chopped into 0.5 cm cubes and placed on a fresh LB agar plate. (3) After 72 hours of incubation at 25 °C, bacterial col-

onies from the crown gall were transferred to a new, fresh LB agar plate. After 48 hours of incubation, a few selected colonies were resuspended in DDH₂O for further experiments.

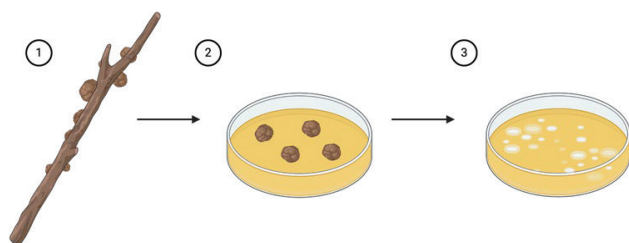


Figure 2: Agrobacterium collection methods. The procedure yielded colonies morphologically consistent with Agrobacterium, which were used for the antimicrobial assays described later.

Herb Extract Preparation:

Figure 3 below shows the herb extract preparation methods for herb extracts. (1) 75 g each of garlic, ginger, cinnamon, and star anise powders was prepared. (2) Each of the powders was mixed separately with 300 mL of DDH₂O. (3) The mixtures were incubated at 4°C for 24 hours for the extraction of active compounds. (4) After incubation, each mixture was filtered. (5) The extracts were stored at -20°C until later use in the experiment.

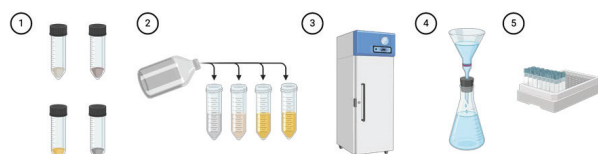


Figure 3: Herb extract preparation. Standardized aqueous extracts (75 g plant material in 300 mL DDH₂O) were produced and stored at -20°C for use in antibiotic selection and seed assays.

Antibiotic Selection Test:

Figure 4 shows the Antibiotic Selection Test procedure. (1) Bacterial cultures were spread on LB agar plates using the spread plate method. (2) Antimicrobial discs were prepared by soaking sterile paper discs (6 mm in diameter) in solutions of different herb extract concentrations (100%, 50%, 25%, 10%, 5%, 1%). An additional paper disc was left unsoaked in extracts and served as a 0% concentration control. Antimicrobial discs were placed onto the surface of each plate using sterile tweezers. (3) The plates were incubated at 25 °C for 24 hours, and a zone of inhibition was observed.

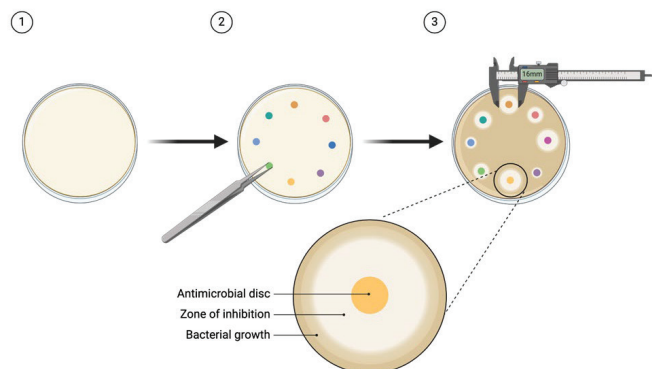


Figure 4: Antibiotic selection test. The disk-diffusion method and concentration series (100–0%) shown here generated the inhibition-zone data used to compare antibacterial potency among extracts.

Plant Seed Germination Test:

Radish seeds were soaked in DDH₂O for 12 hours. Approximately 70 seeds were evenly spaced on the surface of sterile plastic containers. The containers were divided into three treatment groups: one to be sprayed with 25% garlic extract, one with 25% ginger extract, and the other with sterile distilled water as a control. The groups were sprayed at consistent time points (6:00 and 18:00 daily) for 7 days and incubated at 25°C between sprayings.

Data Analysis:

To collect the quantitative data, ImageJ software was used to measure the zone of inhibition diameter in millimeters and root and shoot lengths in centimeters.

Results

Agrobacterium Selection from Wild Woods:

Agrobacterium is known to form white to cream-colored, smooth, convex, glistening, and circular colonies with entire edges that appear mucoid or sticky. The bacterial colonies collected from the wild wood sample also showed similar characteristics, as shown below in the image (Figure 5). The matching characteristics observed suggest that the bacteria sample we obtained was Agrobacterium.



Figure 5: Images of Agrobacterium. Representative colony morphology (white/cream, smooth, convex, mucoid) supported the identification of the isolated strain as Agrobacterium used in the experiments.

Antibiotic Selection Test:

Figure 6 below shows Representative images showing inhibition zones produced by garlic, ginger, star anise, and cinnamon extracts at various concentrations. The diameter of each inhibition zone was measured to evaluate antibacterial effects.



Figure 6: The antibacterial activity of the four herbal extracts. Representative plates show larger inhibition zones for garlic and ginger versus smaller zones for cinnamon and star anise, visually illustrating relative antibacterial potency.

Overall, garlic and ginger solutions were the most effective at killing bacteria, as larger zones with no growth were observed. In contrast, the zones formed by star anise and cinnamon solutions were smaller.

Garlic and ginger had the largest inhibition zone, and they killed bacteria from 25% concentration. Star anise also killed bacteria from 25% concentration, but the size of the inhibition zone was smaller than that of garlic and ginger. Cinnamon showed the smallest inhibition zone, and it could only kill bacteria from 50% concentration (Figure 7).

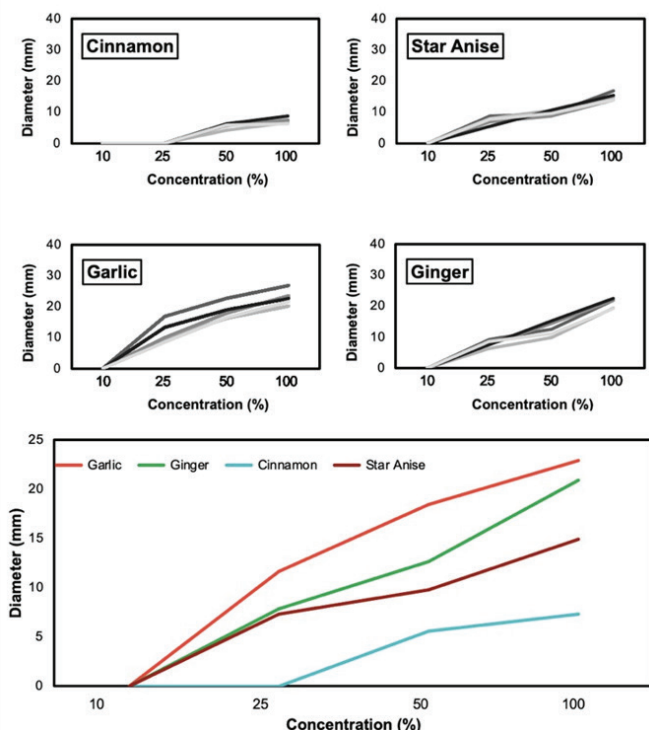


Figure 7: Results of the antibiotic selection test. Quantified inhibition-zone diameters show that garlic and ginger were bactericidal at 25% concentration, star anise was bactericidal at 25% but with smaller zones, and cinnamon required 50% to be bactericidal.

Plant Germination & Growth Test:

In the plant seed germination test, seeds treated with distilled water showed the best overall growth (Figure 8). Seeds treated with the 25% garlic solution were also able to successfully germinate. However, the plant growth was visibly smaller in size compared to that watered with just distilled water. Seeds treated with the 25% ginger solution germinated as well, but showed the smallest growth visually.

Plants grown with distilled water were the longest, with a root length average of approximately 6.08 cm and a shoot length average of approximately 8.55 cm. Next, plants grown with garlic solution were shorter, with a root length average of approximately 3.85 cm and a shoot length average of 4.13 cm. Plants grown with a ginger solution were the shortest, with a root length average of approximately 2.38 cm and a shoot length average of 2.79 cm (Figure 9).



Figure 8: Germination and early growth of seeds under different treatments. Photographs of seedlings reveal that all seeds germinated, but seedlings treated with 25% garlic or 25% ginger displayed visibly reduced growth compared with distilled-water controls.

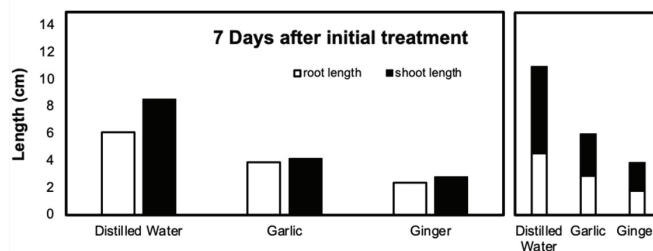


Figure 9: Quantitative comparison of root and shoot lengths. Measured root and shoot lengths confirm that distilled-water controls had the greatest growth (root ~6.08 cm, shoot ~8.55 cm), while 25% garlic and especially 25% ginger produced significantly shorter roots and shoots.

Discussion

Antibiotic Selection Test Analysis:

The results of the antibiotic selection test showed the antimicrobial effects of garlic, ginger, cinnamon, and star anise. These herbal extracts were able to kill bacteria due to their antimicrobial compounds.

Garlic contains allicin, which is an antimicrobial compound that works when raw garlic is crushed or chopped.

Allicin interferes with thiol-containing enzymes in bacteria, disrupting microbial processes such as RNA synthesis and acetyl-CoA formation. Previous studies have shown that garlic is effective against pathogenic bacteria, particularly gram-negative bacteria like *Grobacterium* that are resistant to multiple antibiotics.

Ginger contains compounds like zingiberene, α -curcumene, and zingerone, which can attack the cell membrane of bacteria. This destroys the cell membrane and ultimately leads to leakage of cellular components, inhibiting the growth of bacteria.

Cinnamon contains compounds like trans-cinnamaldehyde and eugenol, which have been shown to have strong antimicrobial properties. Trans-cinnamaldehyde damages the cell membrane of bacteria and disrupts cell division. Eugenol can inhibit bacterial growth by altering the lipid profile and inhibiting the activity of ATPases.

Star anise contains phenolic compounds like shikimic acid, quinic acid, flavonoids, and eugenol. They have antimicrobial properties by damaging bacterial cell membranes and disrupting intracellular enzymes. Specifically, these compounds extracted from star anise have been shown to inhibit biofilm formation and damage pathogenic bacteria.

Additionally, the zones of inhibition were observed for garlic and ginger extracts starting at a concentration of 25%, and these extracts showed larger inhibition zones compared to cinnamon and star anise. This suggests that garlic and ginger are more effective in suppressing bacterial growth than cinnamon and star anise. Garlic and ginger showed superior inhibitory effects on bacteria, likely due to the higher effectiveness of their compounds in damaging bacterial cell membranes.

Plant Seed Germination Test Analysis:

In our experiment, plant growth was significantly lower in treatments with 25% concentrations of garlic and ginger extracts compared to the control group watered with distilled

water. Root and shoot lengths of the plants were all lowest with the garlic and ginger extracts tested.

This result is likely because the concentrations of the garlic and ginger solutions may have caused osmotic stress in the plants. Plant cells need to maintain appropriate turgor pressure and water balance for growth processes like cell division and elongation. However, the high solute levels outside cells from compounds in the extracts would have created an osmotic gradient that draws water out of cells. This dehydration would have disrupted turgor pressure and impaired water and nutrient transport. To re-establish equilibrium, plants would have diverted energy into osmoregulation instead of growth.

Additionally, chemicals in garlic and ginger extracts may have interfered with nutrient uptake.

■ Conclusion

In summation, this study demonstrated both the antimicrobial activity of natural herbal extracts as well as their potential inhibitory effects on plant growth. Our hypothesis that natural herb extracts would suppress bacterial growth without negatively affecting plant growth was partially supported.

The results from the antibiotic selection test confirmed that all extracts - garlic, ginger, cinnamon, and star anise - inhibited the growth of *Agrobacterium* as expected. This validated their antimicrobial properties against pathogenic bacteria.

The plant seed germination test revealed that garlic and ginger extracts, which were the most effective in the antibiotic selection test, did allow successful germination of the plants. However, when compared to the plants treated with distilled water, the minimum concentrations of these extracts required to inhibit bacterial growth also hindered plant growth.

In the field of antibiotics research, this study's results significantly contribute to the existing body of evidence regarding the potential of natural herbal extracts as alternatives to antimicrobial chemicals. The confirmed antimicrobial activity observed in garlic, ginger, cinnamon, and star anise extracts is consistent with previous research about the effectiveness of these natural compounds in treating bacterial infections. Specifically, this study demonstrated the natural herbs' efficacy against *Agrobacterium*, a bacterium known to cause plant diseases.

However, this study also uncovered the inhibitory effects of natural herbal extracts on plant growth. These findings emphasize the need for further exploration and optimization of natural herbal extracts to develop a balance between their antimicrobial properties and their impact on plant growth.

Furthermore, this study addresses the issue of overuse and environmental damage caused by chemically synthesized antimicrobials. Shifting toward natural extracts can help reduce the release of residual antimicrobials into the environment, mitigate ecological disruptions, and combat the development of antimicrobial resistance.

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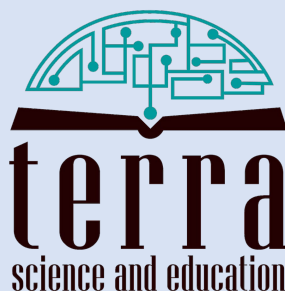
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Taehee is currently a 12th grader at Cheongna Dalton School, South Korea, and enjoys learning about science, math, and engineering. She has developed an interest in biological research on antimicrobials in modern agriculture and medicine. She further pursues her passion for science by leading the Citizen Science Club, which carries out collective volunteer activities to support researchers in multiple fields, including biology, environmental science, and physics.

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