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Early Identification of Autism Spectrum Disorder (ASD), Global Developmental Delay (GDD), and Their Co-Occurrence in Children Aged 2 to 6 Using Machine Learning Analysis on Retinal Images

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Abstract

Background/Objectives: Autism spectrum disorder (ASD) and global developmental delay (GDD) benefit from early identification, yet behavioural assessments are time-intensive, subjective, and often struggle to differentiate these overlapping conditions in young children. Retinal imaging may provide objective, non-invasive biomarkers. **Methods:** In this case–control study, non-mydriatic retinal images from children aged 2–6 years (115 ASD, 151 GDD, 148 co-occurring ASD + GDD, and 1212 age- and sex-matched controls) were analysed using a three-stage machine learning framework combining a deep convolutional neural network and automatic retinal image analysis (ARIA) features with support vector machine classifiers (70% training with 10-fold cross-validation; 30% independent hold-out validation). **Results:** Unconditional cross-validated accuracies were 96.9% for ASD, 96.8% for GDD, and 97.0% for ASD + GDD co-occurrence. In independent hold-out validation, the first-stage model separating children with neurodevelopmental conditions from controls achieved an area under the ROC curve (AUC) of 0.996 (82.8–86.5% unconditional stage-wise accuracies across subgroups). Univariate analyses and Gradient-weighted Class Activation Mapping (Grad-CAM) implicated optic disc, macular, and vascular features, notably increased neuroretina rim area and retinal nerve fibre layer thinning. **Conclusions:** Machine learning retinal image analysis shows promise as a rapid, non-invasive screening

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aid alongside established diagnostic assessments. Furthermore, selectively separating ASD from GDD represents a necessary preliminary step toward future prospective studies evaluating whether retinal biomarkers can identify treatable biological subtypes, such as ASD with cerebral folate deficiency (CFD).

Keywords: autism spectrum disorder; global developmental delay; retinal imaging; machine learning; early identification; pediatric screening

1. Introduction

Autism spectrum disorder (ASD) is a lifelong neurodevelopmental condition characterised by a constellation of features, including difficulties in social interaction and communication (e.g., reduced eye contact and limited facial expression), restricted and repetitive behaviours, highly circumscribed interests, insistence on routines, and atypical sensory reactivity that interferes with daily functioning [1]. Its aetiology remains unclear, and no curative treatment exists, making accurate and timely diagnosis challenging. Although questionnaires and structured interviews are considered the gold standard for screening, many healthcare systems do not routinely implement them. In busy clinical settings, early behavioural signs may be overlooked or misinterpreted—for example, isolative behaviour may be mistaken for shyness [2]. While clinicians can reliably diagnose ASD by age 2, the average age of diagnosis remains between 4 and 5 years due to missed or subtle early symptoms [3]. This delay is clinically significant, as interventions initiated before age 4 (12–48 months) are associated with substantial improvements in cognition, language, and adaptive functioning [4,5] and additional evidence shows that early intervention enhances daily living skills and social behaviour [6]. These findings underscore the critical importance of early identification.

ASD and global developmental delay (GDD) are particularly difficult to differentiate in very young children. GDD is defined as a significant delay in at least two developmental domains—activities of daily living, motor skills, cognition, speech and language, and social–emotional functioning—in children under 5 years of age. Its diagnosis is complicated by a heterogeneous aetiology, with the cause remaining unknown in approximately 62% of cases [7]. Prevalence estimates are imprecise, and diagnostic controversy persists because clinical presentation varies widely and psychometric testing is limited in early childhood. As a result, GDD diagnosis relies on synthesising clinical findings across multiple developmental domains rather than applying a strict objective threshold [8]. After age 5, the term "learning difficulty or disability" is used, with mild learning difficulty (IQ 50–70) affecting 1–2% of children and severe learning difficulty (IQ < 50) affecting 0.3–0.5%, further subdivided into moderate (IQ 35–49), severe (IQ 20–34), and profound (IQ < 20). An objective biomarker capable of distinguishing GDD from ASD would therefore be highly valuable.

Co-occurrences of ASD and GDD are also common. This often reflects shared genetic variants and overlapping neurobiological pathways [9], worsens developmental and functional outcomes, complicates diagnosis, and therefore requires early, multidisciplinary assessment and tailored interventions. Reported co-occurrence rates vary by sample, setting, and assessment methods, but large clinical series report rates of 62.3% of children with GDD meeting ASD criteria; children with lower developmental quotients are more likely to have ASD features [10]. Children with both conditions often exhibit more severe delays across developmental domains, greater social communication challenges, more behavioural difficulties, and increased support needs in educational and daily living contexts. Early identification of co-occurrence is essential to tailor interventions and optimise developmen-

tal outcomes. Early developmental delays (especially in language and social domains) can mask or mimic ASD; standard ASD screening tools may underperform in children with global delays, and clinicians must distinguish qualitative social-communication deficits from *quantitative* delays. This makes early differential diagnosis challenging. So identifying both conditions changes prognosis, educational placement, therapy intensity, and family counselling [11]. For children with GDD, systematic ASD screening is important, and for ASD children and marked global delays, a comprehensive genetic and medical evaluation is recommended because identifying an underlying aetiology can guide surveillance and management [12]. In whichever way we look at the situation, an early non-invasive systematic evaluation of ASD, GDD and possible ASD + GDD co-occurrence will provide valuable guidance to both the parents and the healthcare professionals.

Fundus photography can be an ideal tool for health screening, even in early life. The process is non-invasive and well accepted by the public, and can be performed in community and primary care settings. Researchers have found that it is feasible to obtain nonmydriatic fundus photographs of adequate quality for adult and pediatric patients in non-ophthalmology settings [13–17]. With limited training in fundus camera use, they obtained fundus photos from over 85% of research participants. A previous study has demonstrated that nonmydriatic fundus photographs of adequate quality can be obtained in children as young as 22 months [18].

Researchers have discovered that retinal image analysis is not only for eye examination but also as a marker for cerebral abnormalities. Retinal nerve fibre layer values have been proposed as structural markers of axonal abnormalities and neurodegeneration in multiple sclerosis [19], Alzheimer's disease, and Parkinson's disease [20]. They also found retinal changes in autism. In Gialloreti et al., [21] they evaluated the retinal nerve fibre layer (RNFL) in a group of 24 young ASD adults, with 11 subjects presenting with a diagnosis of "high-functioning autism (HFA)" and 13 with "Asperger Syndrome (AS)", and 24 healthy controls. The study provides evidence of retinal variation across different ASDs, with thinner RNFL in HFA compared to AS or healthy subjects. RNFL thinning is a biological marker of autism pathophysiology; however, other significant factors may also be equally important. In our previous ASD study on students aged 7 to 20, we found significant retinal characteristics between ASD and their age-gender-matched controls. ASD subjects have significantly larger optic disc and optic cup diameters, and we have shown that an objective classification model for ASD is achievable [22].

Cerebral folate deficiency (CFD) is a condition in which folate levels in the central nervous system are inadequate despite normal serum folate. It can result from folate receptor alpha autoantibodies (FRAAs), which block or bind the receptor and impair folate transport across the blood–brain barrier [23,24]. FRAAs are far more common in ASD children than in the general population, with a pooled prevalence of 38% to 70% across ASD cohorts, compared with an estimated 2% to 5% in the general population [24]. Treatment with folinic acid (leucovorin), which bypasses the blocked receptor, improved autism scores in a randomised trial, and binding FRAA has been proposed as a biomarker of leucovorin treatment response in ASD [25,26].

The retina is part of the central nervous system, and retinal cells and retinal microvascular endothelium express folate receptor alpha and the reduced folate carrier, suggesting that retinal folate transport parallels cerebral folate transport, as summarised in a recent review [23]. That paper also reports that disrupted folate and vitamin B12 metabolism, including FRAA-mediated CFD, is associated with thinning of the retinal nerve fibre layer and ganglion cell complex, optic disc changes, and microvascular abnormalities, and untreated CFD in children can cause chorioretinal atrophy [23,27]. The retinal vasculature

serves as a non-invasive proxy for microcirculatory dysfunction elsewhere in the body, and machine learning now makes it possible to read retinal images objectively [28].

These observations led to the proposal that retinal imaging combined with machine learning, which has been used to classify ASD in students aged 7 to 20 years [22] could be extended to identify the subgroup of ASD children who also have CFD, so that folinic acid treatment could begin earlier [23]. That proposal is conceptual: its authors note that no published study has directly correlated retinal imaging metrics with cerebrospinal fluid 5-methyltetrahydrofolate or other direct measures of CFD, and that inferring CFD from retinal imaging remains conjectural [23]. The present study does not directly test this proposal. We used retinal images from children with a diagnosis of ASD, GDD, or both, and from children without these diagnoses, to test whether a machine learning system can identify ASD selectively, including separating it from GDD and from the co-occurrence of ASD and GDD. Selective identification of ASD from retinal images is a necessary but not sufficient first step towards identifying a CFD subgroup within ASD. We did not measure folate status in this cohort, and we did not evaluate the model against any clinical or laboratory measure of CFD, so we present the possible link to CFD as a hypothesis for future testing.

In this study, we aim to develop retinal biological markers, expressed as complex machine-learning composite parameters, that enable accurate classification of ASD and GDD separately, as well as the detection of co-occurring ASD and GDD. This approach could provide an objective, non-invasive screening tool to support early differentiation and risk stratification in young children, used alongside, not in place of, established diagnostic assessments. We also consider how these findings relate to the possibility, proposed in our recent work [23,28] that retinal imaging could help to identify a subgroup of ASD children who have cerebral folate deficiency and may benefit from early treatment.

2. Materials and Methods

This case–control study aims to develop classification models for ASD and GDD in children aged 2–6. We also investigate whether we can separate ASD from the group with co-occurrence of ASD and GDD, and whether a different set of retinal biological parameters is significantly associated with ASD, GDD and the co-occurrence of ASD and GDD separately. We recruited 2212 young children aged between 2 and 6 years for the study. Among them, 115 subjects had a clinical diagnosis of ASD, 151 subjects had GDD, and 148 subjects had both ASD and GDD, all of whom were recruited from special needs schools in Hong Kong. We recruited 1798 controls aged 2–6 from 17 kindergartens; we selected 1212 children with age- and gender-matched cases and controls using 1-to-3 matching overall, except for 30 cases for which we obtained only 1-to-2 matching. After obtaining informed consent from the subjects' parents or guardians, we obtained retinal images using a non-mydriatic Canon EOS 80D fundus camera at 3984 × 2656 resolution (Canon CR-2 Plus AF, Hong Kong SAR, China). We did not capture subjects' identities; we used subject numbers and initials to identify the retinal images and the case report form during data collection and analysis. We conducted the study in compliance with the Declaration of Helsinki. Ethics approval was obtained from the Joint CUHK-NTEC Clinical Research Ethics Committee.

For each analysis, we partitioned the dataset into a 70% training set and a 30% independent hold-out validation set; we performed all model-development procedures exclusively on the training data. In each fold of the 10-fold cross-validation process, we performed preprocessing, feature selection using GLMnet v5.2, and hyperparameter optimisation using only that fold's training data. Samples contained within the validation fold were not used during feature selection, parameter tuning, or model fitting. We did not access the independent hold-out validation dataset until model development was complete.

Explainable AI analysis wasconducted to determine further insights. In addition to our previously developed retinal vessel parameters, we included optical coherence tomography (OCT) parameter estimation, which provides noninvasive retinal structural measures that can serve as biomarkers of neurodegeneration. Recent work highlights retinal ganglion cell and inner plexiform layer changes, macular volume loss, and peripapillary RNFL thinning as potential correlates of central nervous system pathology. Integrating OCT with genetic and neurological assessments could improve phenotyping and monitoring in pediatric neurodevelopmental and neurogenetic disorders.

2.1. Statistical Methods

The statistical methods used in automatic retinal image analysis include fractal analysis, higher-order spectra, and statistical texture analysis. Each method targeted specific retinal image characteristics. We developed the risk assessment model using machine learning and deep learning techniques in MATLAB R2026a. We first applied a deep convolutional neural network, specifically InceptionResNetV2, to retinal images. The outputs were features generated by the 'predictions_softmax' layer, based on pixels associated with the specific outcome status [29]. We also extracted texture, spectral, and fractal-based features associated with the specific outcome using the Automatic Retinal Image Analysis (ARIA) algorithm written in MATLAB [30]. We applied preprocessing techniques, including data augmentation, the synthetic minority oversampling technique (SMOTE), and contrast-limited adaptive histogram equalisation (CLAHE), to increase data diversity and generalisability, address the imbalanced dataset, and enhance retinal images to reduce noise and improve quality. We applied these methods within specified ranges to ensure consistent model performance across imaging conditions and avoid negatively affecting image content [31,32]. We then used the GLMnet approach to select significant features based on penalised maximum likelihood using R v3.6.0 and MATLAB [33,34]. These refined features are highly associated with the specific outcome. To avoid tuning hyperparameters on the entire dataset, which leaks information from the validation sets into the model selection process, we implemented the nested cross-validation hyperparameter tuning within the training folds. In MATLAB, to prevent data leakage during hyperparameter tuning, we used "fitcsvm" with Bayesian Optimisation to obtain the best hyperparameters. Finally, we mapped the machine-learning-extracted features to commonly used retinal characteristics measured in the images using ImageJ v1.53t. This part of the analysis helped establish an "Explainable AI System" to enhance our understanding of retinal vessels and optical coherence tomography (OCT) characteristics that contribute to the classification and identification of specific outcomes. For validation, we employed a 10-fold cross-validation method with a support vector machine (SVM) on test sets not used to train the model [34,35]. We performed this by partitioning the dataset, using a subset to train the algorithm and the remaining subset to test. Each time we ran the cross-validation analysis, we used 10% of the data for testing, which was not included in the training data at all. This method ensures that each run's test set is excluded from the training data, reducing the risk of overfitting and overestimating sensitivity and specificity.

We selected a hybrid CNN + ARIA + SVM framework because it combines high-dimensional image representations generated by InceptionResNetV2 with biologically interpretable retinal features extracted by ARIA. Compared with end-to-end CNN-only approaches, this framework improves interpretability and facilitates explainable AI analyses. We use 10-fold cross-validation on the training dataset to estimate model stability during development, while an independent hold-out validation dataset provides a more conservative estimate of out-of-sample performance. Comparing our framework with previous retinal image classification studies, our three-stage hierarchical classification strategy for

simultaneously distinguishing ASD, GDD, and ASD + GDD co-occurrence has a lot of advantages: Most proposed frameworks with existing machine learning and deep learning methods either use one dimension of the dataset, such as a deep network based directly on pixels, which often lacks interpretation related to biomarkers of the studied diseases, or use multimodal data with different data sources for analysis. In practice, accuracy and effectiveness often trade off. Our approach integrates not only deep-learning-extracted pixel features but also texture-analysis features. Moreover, after confirming the final model, we translate its features directly into biomarkers. A flow chart for the development of the classification model is shown in Figure 1.

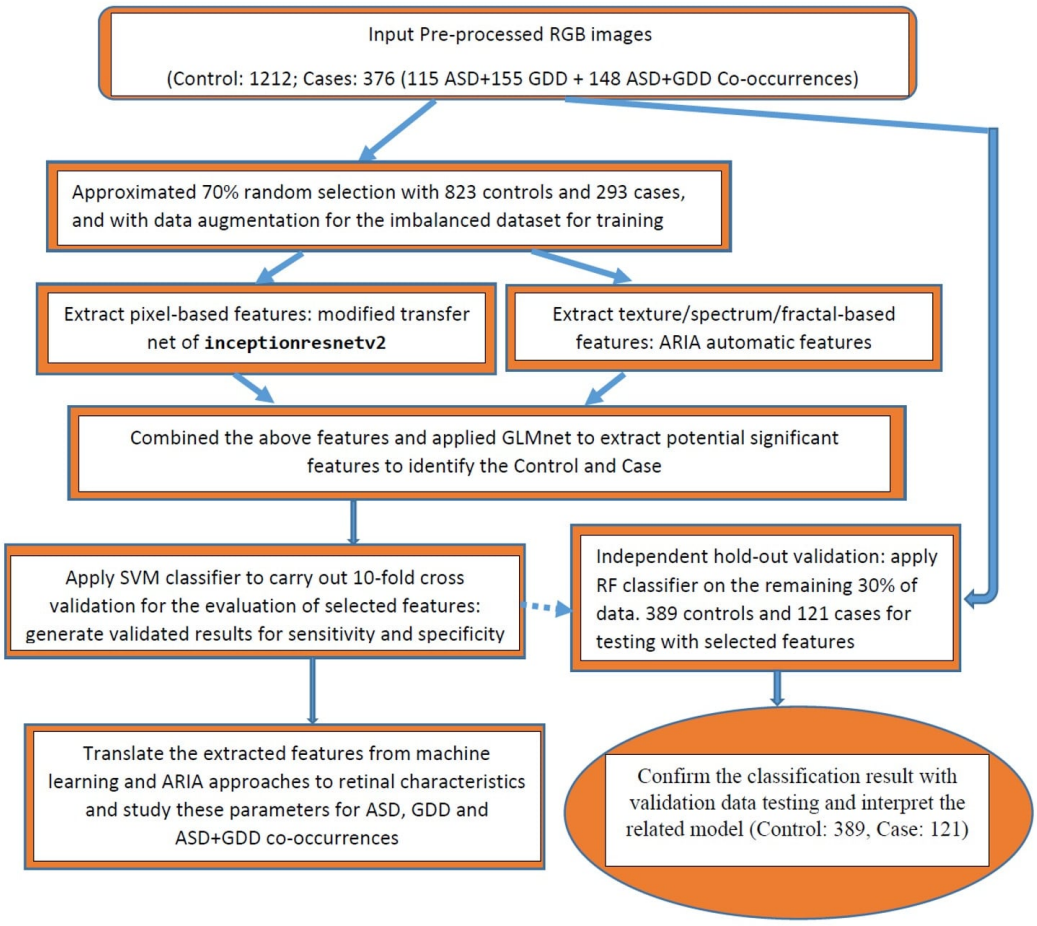


Figure 1. Flowchart for the development of a classification model. RGB–Red, Green and Blueinceptionresnetv2–Pretrained Inception-ResNet-v2 convolutional neural networkGLMnet–Generalised linear model via penalised maximum likelihoodSVM–Support vector machineRF–Random Forest.

Before analysis, we reviewed data distributions and model outputs for plausibility and consistency; however, because the primary focus was machine-learning classification performance, we based inference mainly on performance metrics and their confidence intervals rather than formal hypothesis testing of individual retinal parameters. To account for multiple testing across the three predefined primary classification endpoints, we controlled the overall type I error rate by adjusting the significance threshold as specified in the sample size calculations. We evaluated model performance using sensitivity, specificity, accuracy, and area under the receiver operating characteristic curve (AUC), with corresponding 95% confidence intervals where appropriate. We defined statistical significance as $p < 0.05$ unless otherwise specified, and we considered findings with $0.05 \leq p < 0.10$ borderline significant and interpreted cautiously. We examined demographic characteristics, including

age and sex, as potential confounders and considered them when interpreting retinal feature differences and classification performance. Because the analyses of individual retinal characteristics were exploratory, interpret these findings as hypothesis-generating rather than confirmatory.

2.2. Sample Size

For the classification models, we based our sample size on the estimated 95% confidence intervals for sensitivity and specificity. We adjusted the type I error to 0.008 for multiple endpoints with ASD, GDD and co-occurrence. We expected to achieve approximately 90% sensitivity and specificity for each classification model. Therefore, we planned to recruit at least 140 GDD, 140 ASD, and 280 controls for the classification model. This sample size provides a lower bound of the 95% confidence interval of at least 80%. We calculated the sample size for the three primary classification endpoints (ASD, GDD, and co-occurring ASD + GDD) to determine the minimum number of participants needed to achieve adequate precision for sensitivity and specificity estimates. The final participant distribution differed from the planning assumptions because recruitment reflected the availability of eligible children during the study period. Nevertheless, the overall numbers available for each primary classification endpoint met or exceeded the targeted sample size requirements, and the control sample substantially exceeded the minimum planned recruitment, thereby providing adequate precision for model development and performance evaluation.

3. Results

3.1. Primary Analysis for the Classification of Neurodivergence

In this analysis, we have a total of 1212 controls, 115 ASD alone, 151 GDD alone, and 148 co-occurrences with ASD and GDD (Table 1). In the primary analysis, we used 70% of the data to develop an initial classification model for children with neurodevelopmental conditions, including ASD, GDD and the occurrence group. The training samples include 823 controls and 293 individuals with neurodevelopmental conditions (including ASD, GDD, and the co-occurrence group of both ASD and GDD). The classification results show a sensitivity of 97.5% and a specificity of 99.3% using 10-fold cross-validation. In addition to the 10-fold cross-validation results, we used 30% of the data reserved for an independent hold-out test-set validation. Comparing 389 controls with 121 children with neurodevelopmental conditions, we achieved a sensitivity of 92.6% and a specificity of 97.9%, with mean neurodevelopmental probabilities of 0.08 (n = 389, 95% CI, 0.07–0.09) for controls and 0.72 (n = 121, 95% CI, 0.68–0.75) for cases. The area under the ROC curve was 0.996 (95% CI, 0.992–0.999). Figure 2 shows a box plot of the probability of neurodevelopmental conditions for the independent hold-out validation analysis.

Table 1. Subject baseline characteristics.

Group	Control	ASD	GDD	Co-Occurrence
Female/Male	349/863	28/87	57/94	28/120
Age 2	12(1.0%)	2(1.7%)	4(2.6%)	3(2.0%)
Age 3	114(9.4%)	6(5.2%)	19(12.6%)	9(6.1%)
Age 4	333(27.5%)	39(33.9%)	34(22.5%)	41(27.7%)
Age 5	513(42.3%)	45(39.1%)	67(44.4%)	63(42.6%)
Age 6	240(19.8%)	23(20.0%)	27(17.9%)	32(21.6%)
Total (n)	1212	115	151	148

ASD—Autism spectrum disorder. GDD—Global developmental delay. Co-Occurrence—ASD + GDD.

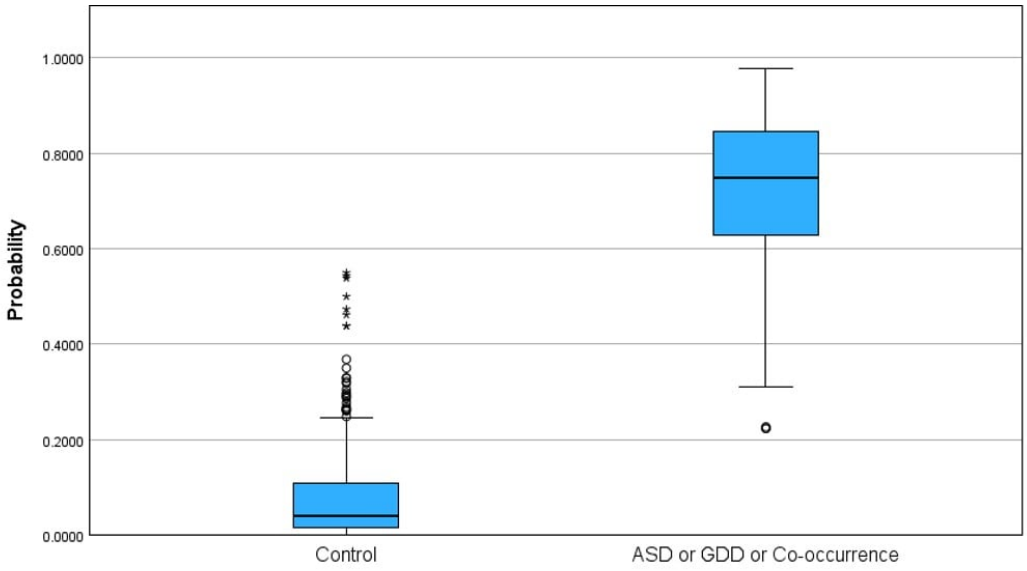


Figure 2. Independent hold-out validation result—classification model for control versus neurodevelopmental cases (ASD, GDD, ASD + GDD). Note: In a box plot, the box length represents the interquartile range (IQR), containing the middle 50% of observations and indicating data variability; the midline inside the box represents the median (50th percentile), showing the distribution's central value. The whiskers extend from the box to the smallest and largest non-outlier observations, reflecting the data's spread. Circles denote outliers, which are observations located more than 1.5 IQRs but less than 3 IQRs beyond the quartiles, while asterisks indicate extreme outliers, which are observations lying more than 3 IQRs from the quartiles.

Among the neurodevelopmental cases, the mean probabilities for ASD alone, GDD alone and the co-occurrence of ASD + GDD are 0.73 (95% CI, 0.673–0.796), 0.72 (95% CI, 0.668–0.777), and 0.69 (95% CI, 0.641–0.742), respectively. These results show that the initial classification model can distinguish the three neurodevelopmental groups from controls similarly, with overlapping confidence intervals.

3.2. Analysis to Separate GDD from ASD and the ASD + GDD Co-Occurrence Group

Among the neurodevelopmental cases in our studies, we want to separate all ASD subjects, including the co-occurrence ASD + GDD cases (n = 263), from GDD (n = 151) subjects as controls. The training algorithm, trained on 70% of the data, achieved 99.7% accuracy for ASD and ASD + GDD co-occurrence cases, versus 99.3% for GDD, using 10-fold cross-validation. The area under the ROC curve was 0.98 (95% CI 0.962–1.0). Figure 3 shows the independent hold-out test-set validation results for 30% of subjects as a box plot of the probability of GDD versus ASD plus ASD + GDD co-occurrence cases. The correct classification rate for GDD is 91.3%, and for ASD plus ASD + GDD is 95.8%. The mean probabilities for GDD and ASD (including ASD + GDD co-occurrence) are 0.34 (n = 46, 95% CI: 0.283–0.391) and 0.84 (n = 71, 95% CI: 0.810–0.864), respectively.

Finally, we developed a classification model to separate ASD-only cases (n = 115) from ASD + GDD co-occurrences (n = 148). We used 70% of the data to train the model, achieving sensitivities of 99.7% for ASD and 99.8% for ASD + GDD in a 10-fold cross-validation analysis. Figure 4 shows independent hold-out test-set validation results for 30% of subjects as a box plot of the probability of ASD versus ASD + GDD co-occurrence, with classification accuracy of 93.3% for ASD and 97.5% for ASD + GDD. The mean probabilities for ASD and the ASD + GDD co-occurrence are 0.20 (n = 45, 95% CI: 0.163–0.237) and 0.72 (n = 40, 95% CI: 0.667–0.768), respectively.

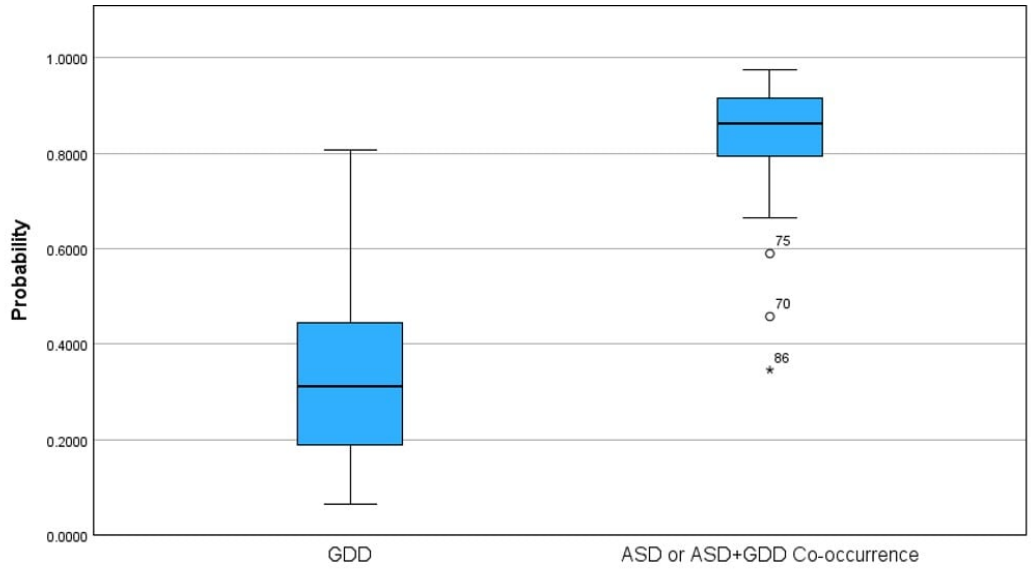


Figure 3. Independent hold-out validation results—classification model for GDD versus ASD and ASD + GDD.

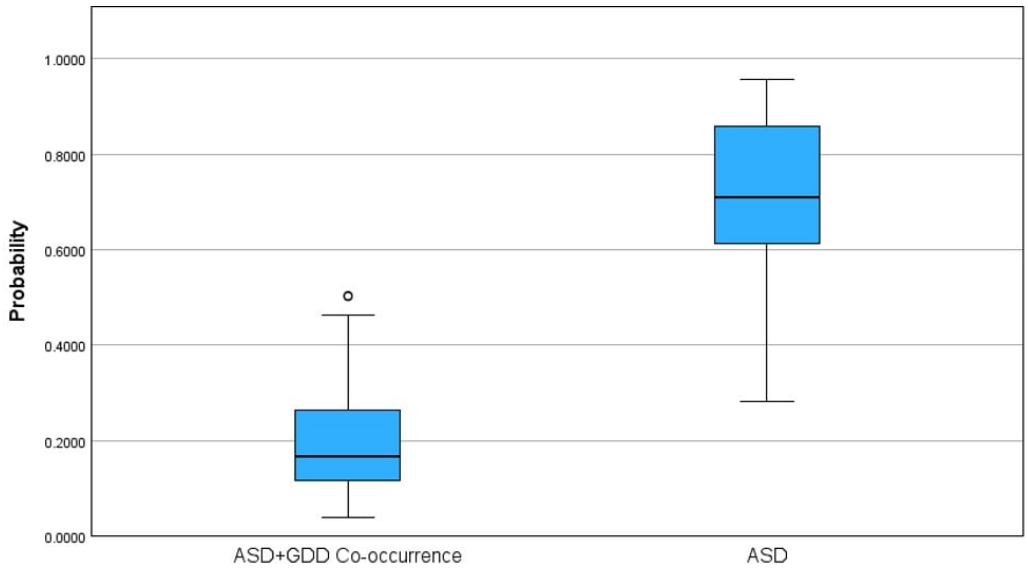


Figure 4. Independent hold-out validation results—classification model for ASD + GDD versus ASD.

3.3. Combining the Conditional Models Results for Practical Application

For the overall application, we use a three-stage analysis to determine the final classification results. We first perform the analysis to evaluate the probability of the neurodevelopmental group, including ASD, GDD, or ASD + GDD co-occurrence (model 1). If the probability is less than 0.5, we classify it as non-neurodevelopmental conditions. The overall application specificity is 99.3% based on 10-fold cross-validation. If the probability exceeds 0.5, we proceed with the analysis to evaluate whether the candidate is classified as GDD versus ASD or ASD + GDD (model 2). If model 2's probability is less than 0.5, we classify the subject as GDD. The unconditional probability of classifying the subject as GDD-only is 97.5% of model 1 times 99.3% of model 2, i.e., 96.8%. If the probability for model 2 exceeds 0.5, we proceed with the analysis to evaluate whether the candidate is ASD or ASD + GDD co-occurrence (model 3). The unconditional probability of classifying the subject as ASD-only is 97.5% of model 1 times 99.7% of model 2 times 99.7% of model 3, i.e., 96.9%. The unconditional probability of classifying the subject as ASD + GDD co-

occurrence is 97.5% of model 1 times 99.7% of model 2 times 99.8% of model 3, i.e., 97.0%. Table 2 summarises the classification performance metrics.

Table 2. Performance metrics of the classifications.

Model Performance	Sensitivity	Specificity	Accuracy	F1-Score	AUC
Stage 1	92.6%	97.9%	96.7%	92.95%	99.6%
Stage 2	95.8%	91.3%	94.0%	95.1%	98.1%
Stage 3	97.5%	93.3%	95.5%	95.12%	99.1%

Sensitivity: The proportion of true positive cases that are correctly identified by the model. Specificity: The proportion of true negative cases that are correctly identified by the model. Accuracy: The overall proportion of correctly classified cases among all cases evaluated. F1-score: The harmonic mean of precision and sensitivity, providing a balanced measure of classification performance. AUC (Area Under the Receiver Operating Characteristic Curve): A measure of the model's ability to distinguish between positive and negative cases across all classification thresholds.

To be more conservative, using the 30% independent hold-out test-set validation data for each of the three-stage analyses, the probability of correctly classifying individuals with neurodevelopmental conditions is 97.9%. The unconditional probability of correctly classifying GDD-only is 92.6% (model 1) times 91.3% (model 2), i.e., 84.5%. The unconditional probability of correctly classifying ASD-only is 92.6% (model 1) times 95.8% (model 2) times 93.3% (model 3), i.e., 82.8%. The unconditional probability of correctly classifying ASD + GDD co-occurrence is 92.6% (model 1) times 95.8% (model 2) times 97.5% (model 3), i.e., 86.5%.

3.4. Explainable AI-Based Retinal Image Analysis

We compared the retinal characteristics (Table 3) and the estimated optical coherence tomography (OCT) parameters (Table 4) among the 115 pure ASD, 151 pure GDD, and 141 ASD + GDD co-occurrence cases, as well as the control groups, to evaluate if there are significant biological differences from a univariate analysis point of view.

Table 3. Retinal characteristics comparing controls vs. ASD vs. GDD vs. ASD + GDD.

Retinal Characteristics	Controls (n = 1212)	GDD (n = 151)	ASD (n = 115)	ASD_GDD (n = 148)	All Neuro-Divergent (n= 414)	p
CRAE_L	13.952	13.901	13.988	13.970	13.950	
CRVE_L	20.652	20.583	20.675	20.678	20.642	
MVasym_L	1.689	1.693	1.687	1.695	1.692	
MAasym_L	1.353	1.350	1.351	1.347	1.349	1
MVangle_L	69.664	69.680	69.571	69.629	69.631	
Mangle_L	72.165	72.447	72.473	72.522	72.481	1
MBCV_L	0.817	0.817	0.819	0.817	0.817	
MBCA_L	0.765	0.766	0.764	0.767	0.766	c
AVR_L	0.675	0.675	0.676	0.675	0.676	
Tortuosity_L	0.345	0.350	0.339	0.341	0.344	
Nipping_L	0.288	0.293	0.289	0.285	0.289	
Hemorrhage_L	0.306	0.309	0.312	0.305	0.308	
Aocclusion_L	0.080	0.080	0.083	0.078	0.080	
Exudates_L	0.221	0.217	0.222	0.216	0.218	
CRAE_R	13.735	13.651	13.738	13.698	13.692	
CRVE_R	20.401	20.303	20.371	20.318	20.327	1
MVasym_R	1.687	1.694	1.684	1.691	1.690	
MAasym_R	1.334	1.338	1.335	1.336	1.336	
MVangle_R	70.202	70.305	70.156	70.310	70.265	
Mangle_R	73.153	73.133	73.108	72.917	73.049	

Table 3. *Cont.*

Retinal Characteristics	Controls (n = 1212)	GDD (n = 151)	ASD (n = 115)	ASD_GDD (n = 148)	All Neuro-Divergent (n= 414)	p
MBCV_R	0.826	0.828	0.826	0.825	0.827	2
MBCA_R	0.766	0.767	0.765	0.768	0.767	
AVR_R	0.674	0.672	0.674	0.674	0.673	
Tortuosity_R	0.368	0.373	0.370	0.369	0.371	
Nipping_R	0.294	0.299	0.299	0.305	0.301	1
Hemorrhage_R	0.333	0.336	0.349	0.340	0.341	1
Aocclusion_R	0.074	0.074	0.081	0.073	0.076	3
Exudates_R	0.201	0.206	0.217	0.211	0.211	1

Note: A numeral in the p column denotes $p < 0.05$ and a letter denotes $0.05 < p < 0.1$ for the following comparisons: 1/a, controls versus all neurodevelopmental cases; 2/b, GDD versus ASD + GDD; 3/c, ASD versus ASD + GDD; 4/d, ASD versus GDD.

Table 4. OCT characteristics comparing controls vs. GDD vs. ASD vs. ASD + GDD.

OCT Characteristics	Controls (n = 1212)	GDD (n = 151)	ASD (n = 115)	ASD_GDD (n = 148)	All Neuro-Divergent (n= 414)	p
AverageGCIPL_thickness_L	76.604	76.519	76.570	76.686	76.593	
MinGCIPL_thickness_L	69.680	69.491	69.542	69.801	69.616	
Radii_L	133.850	134.364	133.234	134.035	133.932	
Disc_di_L	52,929.207	53,451.546	52,573.094	53,233.276	53,129.503	3
Cup_di_L	31,432.334	31,356.668	30,623.771	32,155.797	31,438.765	
Rim_di_L	25,492.221	25,783.746	25,416.169	25,395.617	25,542.890	
CDR_L	0.560	0.557	0.556	0.562	0.559	b, c
RDR_L	2.007	2.034	1.964	2.010	2.006	
RNFL_mthickness_L	93.752	93.801	94.058	93.851	93.890	
Rim_area_L	2.119	2.168	2.246	2.205	2.203	1
Disc_area_L	2.168	2.160	2.148	2.184	2.165	3, b
averageC_Dratio_L	0.633	0.630	0.624	0.649	0.635	3, b
verticalC_Dratio_L	0.540	0.541	0.540	0.540	0.540	
Cup_volume_L	0.410	0.408	0.405	0.410	0.408	
AverageGCIPL_thickness_R	72.473	72.353	72.199	72.230	72.266	
MinGCIPL_thickness_R	63.995	63.528	63.689	63.594	63.596	a
Radii_R	143.663	143.934	143.862	143.651	143.813	
Disc_di_R	57,930.543	57,957.703	57,966.141	57,536.580	57,809.500	
Cup_di_R	30,957.986	30,645.771	30,883.641	30,903.372	30,803.935	
Rim_di_R	30,976.267	31,053.750	31,204.258	31,056.518	31,096.547	
CDR_R	0.512	0.507	0.511	0.513	0.510	
RDR_R	5.981	5.705	5.780	6.443	5.990	
RNFL_mthickness_R	88.589	87.956	87.739	88.181	87.976	1
Rim_area_R	1.158	1.157	1.149	1.158	1.155	c, d
Disc_area_R	2.191	2.189	2.190	2.186	2.188	
averageC_Dratio_R	0.633	0.629	0.630	0.632	0.631	a
verticalC_Dratio_R	0.601	0.600	0.603	0.601	0.601	
Cup_volume_R	0.436	0.437	0.440	0.435	0.437	

Note: A numeral in the p column denotes $p < 0.05$ and a letter denotes $0.05 < p < 0.1$ for the following comparisons: 1/a, controls versus all neurodevelopmental cases; 2/b, GDD versus ASD + GDD; 3/c, ASD versus ASD + GDD; 4/d, ASD versus GDD.

In Table 3, significant univariate retinal characteristics between controls and all neurodevelopmental cases include asymmetry, bifurcation angle, CRVE, nipping, haemorrhage, occlusion, and exudates. The bifurcation coefficient differed significantly between GDD and the co-occurrence, and occlusion differed significantly between ASD and the co-occurrence. Among the OCT parameters in Table 4, rim area increase and retinal nerve fibre layer thinning are the two most obvious characteristics that distinguish neurodivergent cases from controls. Optic disc diameter, optic disc area, and cup-to-disc ratio are significant parameters that distinguish ASD from co-occurring ASD + GDD cases. It is noted that the difference between ASD and GDD is not obvious from a univariate analysis perspective.

We have reorganised the significant variables into Table 5, which summarises the fundus and OCT characteristics that provide a meaningful means of distinguishing each case type. It is presented as a significant characteristic for distinguishing between types when $p < 0.05$, and as a borderline significant characteristic when the calculated p values are between 0.05 and 0.1.

Table 5. OCT and retinal characteristics that significantly distinguish neurodivergences.

	Significant Variables ($p < 0.05$)		**Borderline Significant Variables ($0.05 < p < 0.01$)**	
	Fundus	**OCT**	**Fundus**	**OCT**
Neurodivergence v. Control	MAasym_L Mangle_L CRVE_R Nipping_R Hemorrhage_R Exudates_R	Rim_area_L RNFL-mt_R		MinGClPL_t_R average_Dratio_R
GDD v. ASD + GDD	MBCV_R			CDR_L Disc_area_L average_Dratio_L
ASD v. ASD + GDD	Aocclusion_R	Disc_di_L Disc_area_L averageC_DratioL	MBCA_L	CDR_L Rim_area_R
ASD v. GDD				Rim_area_R

We also apply a combination of gradient and activation maps (Grad-CAM) to visualise which parts of an image are most important to the classification decision in our analysis. Grad-CAM provides a class-specific heatmap showing where the convolutional neural network looks when making a prediction. It does this by combining gradients (which indicate how important each feature map is for the classification) with activations (which indicate what the feature maps detected). The result is a localisation map highlighting the image regions most influential for the model's decision. An example of the control in Figure 5 shows that both macular and optic disc regions are important. Corresponding examples for the GDD, ASD, and ASD + GDD co-occurrence groups are shown in Figures 6–8, respectively.

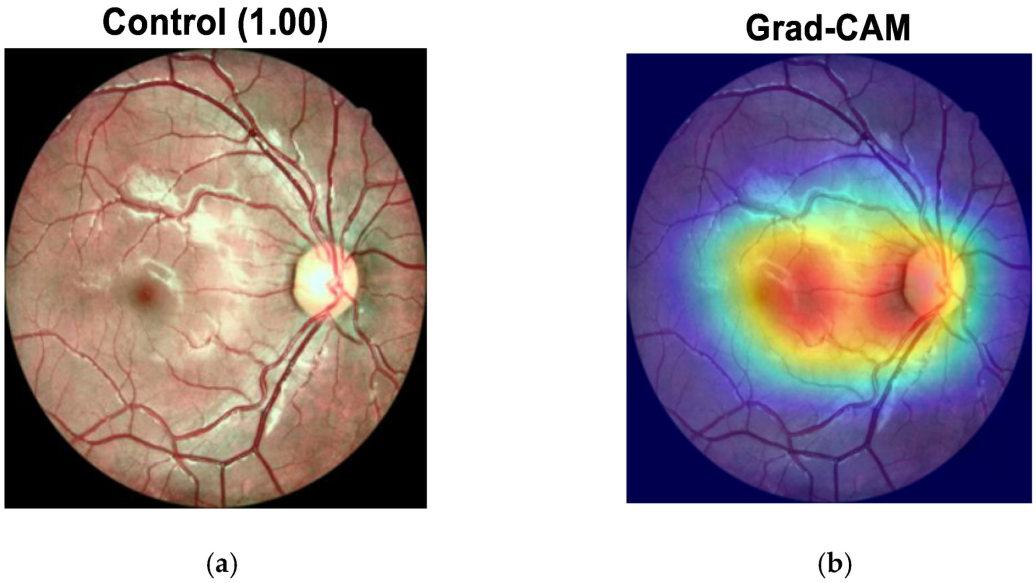
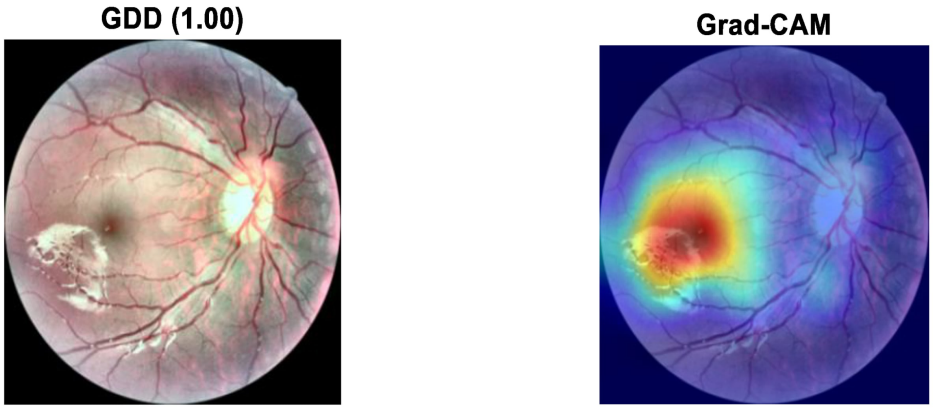
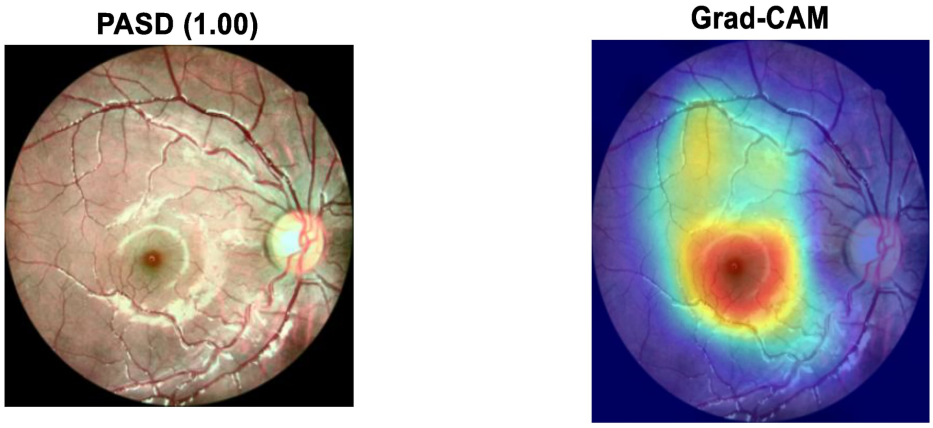


Figure 5. Grad-CAM analysis for the control group. (**a**) Retinal fundus image labelled with the predicted class and score; (**b**) corresponding Grad-CAM heatmap.



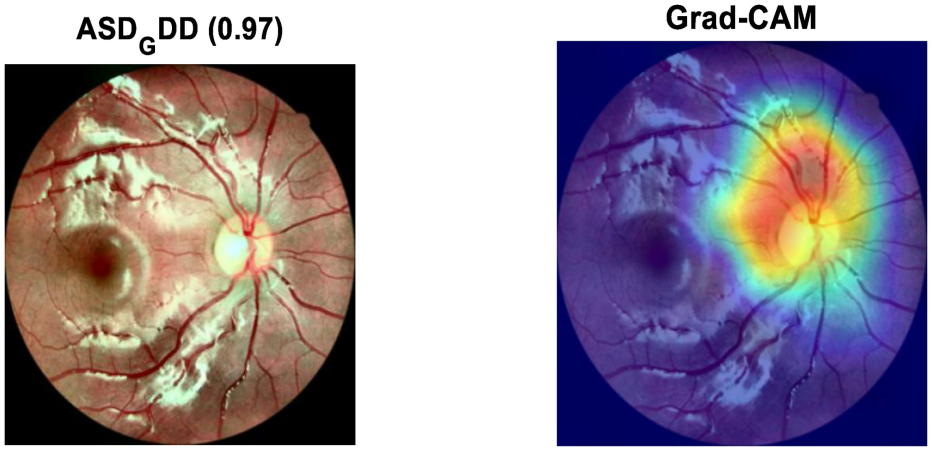
(**a**) (**b**)

Figure 6. Grad-CAM analysis for the GDD group. (**a**) Retinal fundus image labelled with the predicted class and score; (**b**) corresponding Grad-CAM heatmap.



(**a**) (**b**)

Figure 7. Grad-CAM analysis for the ASD group. (**a**) Retinal fundus image labelled with the predicted class and score; (**b**) corresponding Grad-CAM heatmap.



(**a**) (**b**)

Figure 8. Grad-CAM analysis for the ASD + GDD co-occurrence group. (**a**) Retinal fundus image labelled with the predicted class and score; (**b**) corresponding Grad-CAM heatmap.

The results show a clear difference between controls and all neurodevelopmental cases, and significant univariate parameters among ASD-only, GDD-only and ASD + GDD are also detected. However, the difference between ASD-only and GDD-only is not apparent in either the univariate or Grad-CAM analyses.

4. Discussion

4.1. Participant Selection Provides a Consistent Dataset for Analysis

The neurodevelopmental cases in this study were recruited from special needs schools in Hong Kong and are of high quality because they are identified within a tightly coordinated, multi-agency system designed to ensure accuracy, consistency, and early identification. The developmental concerns of young children are first detected through universal surveillance in Maternal and Child Health Centres and schools, after which suspected cases undergo comprehensive, multidisciplinary assessment at the Department of Health's Child Assessment Service (CAS) [36]. CAS employs structured nurse-led screening, standardised developmental tools, and a triage system that prioritises children with significant delays, ensuring timely and appropriate evaluation. Diagnostic decisions are further strengthened through continuous communication with therapists, teachers, and service operators using a response-to-intervention model, along with systematic follow-up, questionnaires, and Pre-Primary One assessments for children with complex needs. In parallel, the Social Welfare Department's Central Referral System for Rehabilitation Services (CRSRehab-PS) [37] provides a transparent, standardised mechanism to verify diagnoses, coordinate referrals, and match children with suitable preschool rehabilitation services. All referrals require certification by qualified paediatricians or psychologists, and placement decisions follow strict procedural rules, regular data updates, and ongoing monitoring of developmental progress. Together, CAS and CRSRehab-PS form an integrated, quality-controlled pathway that ensures ASD and GDD classifications are based on robust professional assessment, consistent criteria, and longitudinal validation across health, education, and social welfare sectors.

4.2. Our System Accurately Distinguishes Between ASD, GDD, and ASD + GDD

The primary key of this study is the early detection of ASD and GDD so that early interventions can be considered. The ability to identify ASD and GDD early is vital because it fundamentally changes developmental trajectories and family outcomes. Both conditions affect communication, cognition, and adaptive functioning, but when identified early, typically before age 4, children can access targeted interventions during the brain's most plastic developmental period. Evidence shows that early behavioural and educational therapies significantly improve language, social engagement, and daily living skills, while reducing long-term disability and dependence [38]. From a systems perspective, early detection also enables timely triage and coordinated care across a multidisciplinary network, linking Maternal and Child Health Centres, the Child Assessment Service, and the Social Welfare Department's rehabilitation programmes. This integration ensures that children receive appropriate support before school entry, preventing delays in therapy and educational placement. Moreover, early identification reduces family stress by providing clarity, structured guidance, and access to parent-training programmes that enhance caregiving capacity. In short, detecting ASD and GDD early allows clinicians to intervene when neurodevelopment is most malleable, optimises resource allocation, and empowers families to participate actively in their child's growth and learning—transforming what might otherwise be lifelong challenges into manageable developmental pathways.

In our study, clarifying ASD + GDD co-occurrence in the classification process is essential because our analysis showed that it is not simply an additive combination of two conditions—it represents a distinct neurodevelopmental profile with different clinical

implications, service needs, and developmental trajectories. Treating ASD + GDD as a single homogeneous group with either ASD or GDD obscures important differences that directly affect diagnosis, intervention planning, and resource allocation.

First, co-occurrence is common. Our study shows that 148 out of 414 neurodivergent children (≈36%) had both ASD and GDD, and external literature reports that 62.3% of children with GDD meet ASD criteria. Because the overlap is so large, failing to distinguish the combined phenotype risks misclassification and reduces the accuracy of any screening or diagnostic model. Our results clearly show that ASD-only, GDD-only, and ASD + GDD have distinct retinal signatures, and machine-learning models can separate them with high accuracy. This means the co-occurrence group is not a statistical blur—it is a biologically meaningful category.

Second, ASD + GDD cases typically present with greater severity, including more profound language delays, more behavioural challenges, and higher support needs in daily living. These children require varying levels of intervention, different educational placement considerations, and often earlier access to multidisciplinary services. If the classification model does not explicitly identify the co-occurrence group, these children may be under-referred or placed in programmes that do not match their needs.

Third, from a systems perspective—especially in Hong Kong's CAS → CRSRehab-PS → Early Education and Training Centre (EETC)/On-site Pre-school Rehabilitation Services (OPRS)/Special Child Care Centre (SCCC) pathway—accurate differentiation directly influences triage priority, service type, and waitlist management. ASD + GDD children are more likely to require SCCC or intensive OPRS support, and misclassification could delay access to appropriate rehabilitation services.

Finally, clarifying the co-occurrence group strengthens the scientific value of the retinal biomarker work. Demonstrating that ASD + GDD has a distinct retinal phenotype supports the biological plausibility of retinal imaging as an objective tool for early neurodevelopmental differentiation. This is especially important because behavioural assessments in very young children often struggle to disentangle ASD features from global delays. In conclusion, distinguishing ASD + GDD is crucial because it improves diagnostic precision, ensures appropriate intervention pathways, enhances service planning, and validates the biological relevance of the machine-learning retinal markers.

4.3. Early and Accurate Detection Enables Optimised Treatment for Each Child

From the perspective of intervention, early identification of ASD, GDD, and especially the ASD + GDD co-occurrence fundamentally reshapes the intervention pathway because each group has different developmental profiles, different risks, and different service needs. When these distinctions are made early and accurately, intervention recommendations become more targeted, more intensive where needed, and more effective overall.

- Early identification allows interventions to match the child's true developmental profile. For ASD-only children, they require specialised social-communication interventions, behavioural therapy, parent-mediated programmes, and structured teaching. Language support focuses on pragmatics, joint attention, and social reciprocity. Motor or cognitive delays may be mild or absent, so therapy is more behavioural and communication-focused. GDD-only children require broad developmental stimulation across multiple domains: cognition, motor skills, language, and adaptive behaviour. Interventions emphasise global skill-building, structured play, and early education. Therapy intensity depends on the number of domains affected, not on autism-specific needs. These two groups already diverge significantly in recommended intervention types.
- ASD + GDD co-occurrence requires a different level and combination of support. Children with both ASD and GDD show more severe delays across multiple domains, have

greater behavioural challenges, require higher-intensity, multidisciplinary intervention, and often need earlier access to specialised services (e.g., SCCC, intensive OPRS, or HA multidisciplinary therapy). If ASD + GDD is not identified as a distinct group, these children risk being under-referred, placed in programmes that are too mild, or given interventions that do not match their complexity. Early clarification ensures they receive the right intensity, setting, and combination of therapies.

- Early identification improves family guidance and expectation management. Parents of ASD + GDD children need a clearer understanding of the prognosis, more structured parent-training, and support for managing both behavioural and cognitive challenges, and guidance on long-term educational planning. Families of ASD-only or GDD-only children need different information and expectations.
- Early identification strengthens intervention planning before primary school. Pre-Primary One (Pre-P1) assessment and school placement decisions depend on cognitive level, adaptive functioning, behavioural profile, and autism-related needs. Accurate early classification ensures appropriate SEN support in mainstream schools, appropriate special school placement when needed, and a smoother transition with fewer disruptions.
- Using the results of our study, distinguishing ASD, GDD, and ASD + GDD enhances clinical utility. Our results show that ASD, GDD, and ASD + GDD each have distinct retinal-biological signatures and can be classified with high accuracy. This means the tool is not just detecting "neurodivergence"—it can differentiate among types of neurodevelopmental conditions, which is exactly what we urgently need for children under 4. This differentiation directly affects intervention recommendations because ASD-only children need autism-specific interventions (e.g., social-communication therapy, structured behavioural programmes, parent-mediated interaction training). GDD-only children need global developmental stimulation (e.g., cognitive enrichment, motor training, early education support). ASD + GDD children need BOTH—and at higher intensity (e.g., multidisciplinary therapy, more hours, specialised settings like SCCC, earlier placement). Without identifying the co-occurrence, clinicians risk under-prescribing or mis-prescribing interventions.
- Early identification prevents "diagnostic overshadowing". In young children, as GDD can mask ASD, ASD can distort developmental testing, and co-occurrence can be mistaken for "severe ASD" or "severe GDD". The AI-based retinal image analysis classification model helps avoid this by providing an objective biological marker, reducing reliance on behavioural interpretation alone. This is especially important because ASD screening tools underperform in children with GDD, and GDD can delay ASD diagnosis by years. Co-occurrence is often missed until school age, and early clarity enables timely, appropriate intervention.

Thus, early identification plus clear differentiation improves outcomes because the brain is most plastic between 12 and 48 months. Early therapy improves language, cognition, adaptive skills, and social behaviour. Parents can be empowered earlier. Children enter primary school with better readiness. Service systems can allocate resources more efficiently. The AI-based retinal image analysis approach adds something the system currently lacks: an objective, non-invasive, scalable biomarker that can differentiate ASD, GDD, and ASD + GDD before behavioural symptoms fully emerge.

4.4. Appropriate Early Intervention for 'Gifted' ASD Children

Educational intervention plays a special role for ASD children with gifted and talented profiles. Early identification of ASD and GDD is crucial, but equally important is recognising that not all autistic children present with delays. Some show advanced abilities, "spiky" de-

velopmental profiles, or asynchronous development, where high-level strengths coexist with social-communication challenges. These children are often described as high-functioning or ASD with domain-specific talents (e.g., visual-spatial reasoning, memory, pattern recognition, early reading, mathematics, music, etc.) For these children, educational intervention plays a uniquely important role that differs from both ASD-only and GDD-only pathways.

- Gifted autistic children are often missed, misunderstood, or misplaced. Without early identification, their strengths can mask their challenges—or their challenges can overshadow their strengths. This can lead to academic under-stimulation, behavioural issues from boredom or frustration, misinterpretation as "noncompliant" or "oppositional", and placement in programmes that do not match their cognitive needs. Early recognition allows educators to design dual-pathway interventions that support both strengths and challenges.
- Their learning profile is highly uneven—requiring tailored educational planning. Gifted autistic children often show advanced reasoning but weak executive functioning, strong memory but poor social inference, high verbal ability but difficulty with pragmatic language, exceptional visual-spatial skills but challenges with flexibility. This "spiky profile" means standard ASD interventions are insufficient and standard gifted programmes are inappropriate. Educational intervention must therefore build on strengths, support areas of vulnerability, and prevent emotional/behavioural fallout from mismatch.
- Early educational intervention prevents secondary problems. Without appropriate educational support, gifted autistic children are at high risk of anxiety, school refusal, depression, perfectionism, social isolation, and underachievement. Early identification can prevent these when it leads to enrichment, acceleration in specific domains, social–emotional coaching, and structured support for executive functioning.
- Educational intervention is the *primary* driver of outcomes for ASD–Gifted profiles. Unlike ASD + GDD, where therapy intensity and multidisciplinary support dominate, ASD–Gifted profiles benefit most from educational design, including strength-based learning and using their interests and talents as motivators. They also benefit from curriculum differentiation by providing advanced content in their areas of strength and flexible pacing, which allows acceleration in some subjects and support in others. Explicit teaching of social–emotional skills is important for them because cognitive ability does not eliminate social-communication challenges. Executive-function support helps them manage perfectionism, organisation, and transitions, which may lead to better outcomes.
- Early classification prevents misplacement in the wrong service pathway. Since ASD–Gifted children require mainstream placement with SEN support and enrichment programmes, teacher training becomes exceptionally important. We should also avoid overly restrictive environments. Early identification ensures they are not placed in settings that limit their potential.

In summary, educational intervention has a special role for ASD–Gifted because these children have unique strengths that must be nurtured. They have vulnerabilities that require targeted support and are at high risk of misdiagnosis and misplacement. They benefit more from educational design than from traditional therapy. Early identification is needed to prevent long-term emotional and academic difficulties. Educational intervention is not just helpful; it is central to their developmental trajectory.

4.5. Facilitating Early Treatment of Cerebral Folate Deficiency

Folate receptor alpha autoantibodies (FRAAs) are found in 38% to 70% of ASD children across published cohorts [24]. FRAAs can block folate transport into the brain and

cause cerebral folate deficiency (CFD), a condition in which folate levels in the brain and cerebrospinal fluid 5-methyltetrahydrofolate (5-MTHF) are low despite normal blood folate. This leads to Impaired methylation, reduced neurotransmitter synthesis, neuroinflammation, and, in some cases, developmental regression. CFD is treatable with levoleucovorin (L-folinic acid), and early identification is clinically more advantageous [39]. Levoleucovorin is the active, bioavailable form of folinic acid. It bypasses the blocked folate receptor, restores folate levels in the CNS, supports methylation and neurotransmitter pathways, and improves neurodevelopmental functioning in children with CFD. It is not a general ASD treatment and is targeted for children with biological evidence of folate transport dysfunction. Clinical studies (including controlled trials) have shown improvements in core ASD-related domains, including language (especially expressive and receptive), social communication, and attention and cognitive flexibility. Children who test positive for FRAA tend to show the strongest response [40].

Children with CFD may be confused as having global developmental delay, even without classic ASD features. These children may be misclassified as "non-specific GDD" unless CFD is being considered. For the ASD + GDD co-occurrence, this is particularly important. They often have more severe neurodevelopmental impairment, and it is plausible, although it was not examined in this study, that metabolic or immune abnormalities, including CFD, are more frequent in this group. They may be the most likely to be overlooked because delays are attributed to "global delay" rather than a treatable metabolic issue. Early identification of ASD + GDD co-occurrence could prompt clinicians to consider metabolic evaluation for CFD, but this study did not test whether such evaluation would identify CFD or enable targeted treatment.

The AI-based retinal image analysis classification framework could become useful if CFD proves to be more prevalent in ASD with regression, ASD + GDD co-occurrence cases, and ASD with severe language delay, which this study did not assess. Our model has not been evaluated against FRAA or CSF 5-MTHF measurements and cannot currently identify children with CFD. If a retinal model were validated against such measurements, it might help to flag children for FRAA testing, with CSF 5-MTHF testing considered when indicated, so that folinic acid treatment could be initiated sooner. While folinic acid is widely studied, decisions about testing and treatment must be made by qualified clinicians. It is noted that not all autistic children have CFD. Testing for FRAA or CSF folate is required before considering treatment, and the treatment response varies. It is not a replacement for behavioural or educational interventions, but for the subgroup with CFD, it can be transformative.

Early identification of ASD, GDD, and ASD + GDD could, if followed by appropriate biochemical testing, help clinicians to detect treatable biological subtypes such as cerebral folate deficiency. Levoleucovorin plays a special role because it can correct a metabolic abnormality that contributes to developmental delay, language impairment, and autistic features. Recognising the ASD + GDD group is especially important because they may have the highest likelihood of underlying metabolic dysfunction and the greatest potential benefit from targeted treatment.

This study does not provide direct clinical or laboratory evidence for the retinal imaging approach to detecting CFD described in our recent review of folate and vitamin B12 metabolic dysfunction [23]. That review proposed that, because folate transport into the retina parallels folate transport into the brain, retinal imaging combined with machine learning could be developed to detect CFD, and it noted that machine learning had not yet been applied to retinal images for this purpose. That review also described the link between retinal and cerebral folate transport as biologically plausible but not directly demonstrated. The results reported here show that retinal images from children aged 2 to 6 years contain

information that a machine learning system can use to identify ASD selectively, separating it from GDD and from the co-occurrence of ASD and GDD, with cross-validated accuracies of 96.8% to 97.0%. This is a necessary but not sufficient step towards identifying CFD.

The retinal features that distinguished neurodivergent children from controls in this study also belong to the categories of retinal change described in folate-related disease. We found increased optic disc rim area and thinner retinal nerve fibre layer, together with differences in vessel measures such as the central retinal vein equivalent and arteriolar nipping. A recent review reports thinning of the retinal nerve fibre layer and ganglion cell complex, optic disc changes, and microvascular abnormalities in disorders of folate and vitamin B12 metabolism, including FRAA-mediated CFD [23], and untreated paediatric CFD has been associated with chorioretinal atrophy [27]. The vascular findings are also consistent with the view that the retina reflects microcirculatory dysfunction elsewhere in the body, and that treatment with B vitamins can restore microcirculation in some conditions [28]. These are similarities in the type of retinal feature affected, not evidence of a shared mechanism. We did not compare these parameters with folate status in our cohort, nor did we compare the direction and magnitude of CFD changes with ours. No study has yet directly correlated retinal imaging metrics with cerebrospinal fluid 5-MTHF or other direct measures of CFD [23].

A possible, but untested, clinical pathway for ASD children has two steps. First, a retinal screen such as the one reported here could flag young children who are likely to be diagnosed with ASD, or ASD together with GDD, for referral to formal diagnostic assessment. Second, children identified in this way could be tested for FRAA and, where indicated, treated with folinic acid, which has improved verbal communication and autism scores in randomised trials [25,40]. Both steps depend on evidence that this study does not provide. The retinal images analysed here were not paired with FRAA results, folate or homocysteine levels, or cerebrospinal fluid folate measurements, so we cannot say whether any subgroup within the ASD or ASD + GDD groups had CFD, and the proposal that retinal imaging can identify such a subgroup remains a hypothesis. Testing it will require a prospective cohort in which retinal images are collected together with FRAA status and other folate biomarkers, so that a model can be trained and validated against a biochemical reference standard, as set out in our proposed framework [23]. Such a study could also test whether CFD is more frequent in the ASD + GDD group, as suggested above.

5. Conclusions

Our study shows that retinal biomarkers, analysed with our AI framework, can accurately differentiate ASD, GDD, and ASD + GDD co-occurrence in young children. The high quality of our dataset provides a robust foundation for establishing that these three neurodevelopmental profiles are not only clinically distinct but also biologically separable using non-invasive retinal imaging. Our findings carry important translational value. By accurately distinguishing these three conditions, our model addresses a gap in early paediatric screening, as current behavioural assessments offer limited value for children under 4, delaying appropriate intervention. Our model may serve as a screening aid that flags and distinguishes these conditions, supporting timely referral for formal diagnostic assessment and, if a diagnosis is confirmed, early intervention, services, and family guidance. It is not intended at this time to replace established diagnostic assessments. These findings do not provide direct evidence for Ayoub and Brown's [23] proposal that retinal imaging can detect cerebral folate deficiency (CFD); at most, they show that retinal separation of closely related neurodevelopmental groups, a prerequisite step, is feasible. In that proposal, folate receptor alpha autoantibodies impair folate transport into both the brain and the retina, so CFD might leave a measurable retinal signature, but no study has yet correlated retinal

imaging with direct measures of CFD [23], and our cohort had no folate, folate receptor autoantibody, or cerebrospinal fluid measurements. Our model separated ASD from GDD, and from ASD with GDD, which are closely related phenotypes that are difficult to tell apart in young children.

Therefore, early and accurate differentiation may help to prioritise children for further evaluation and, if future studies validate the approach, for testing for treatable biological subtypes such as cerebral folate deficiency, in which early vitamin replacement may provide lasting benefit. Taken together, our findings support the retinal biomarker-AI framework as a potential scalable, objective screening tool alongside existing behavioural and clinical assessment paths. It is not yet a replacement for established diagnostic assessments, and prospective studies are needed to establish its effect on diagnostic precision, individualised intervention, and outcomes for a heterogeneous population of neurodivergent children. CFD is an important target because folate receptor autoantibodies, one cause of CFD, are found in 38% to 70% of ASD children across published cohorts [24] and folinic acid has improved autism scores and verbal communication in randomised trials [25,40]. Evidence on maternal folate receptor autoantibodies and prenatal autism risk is limited. One small study of 11 pregnancies selected for markedly increased nuchal translucency found autism in all four children of autoantibody-positive mothers and in one of seven children of autoantibody-negative mothers [41]. A single-centre pilot randomised study in autoantibody-positive women, analysing 18 of 29 randomised participants per protocol, found autism in 1 of 10 children whose mothers received folinic acid and in 5 of 8 whose mothers received folic acid [42]. Both studies, from the same group, describe their findings as preliminary, and neither examined retinal imaging, so they do not show that retinal imaging can predict prenatal autism risk. Our cohort comprised children aged 2 to 6 years with no maternal or prenatal data, so we provide no new evidence on prenatal prediction. Ayoub and Brown [23] proposed that retinal imaging might eventually support screening in autoantibody-positive or high-risk pregnancies, but they describe this as a working hypothesis and a research direction rather than a validated application. Testing this would require prospective studies that pair retinal images with folate receptor autoantibody results and offspring outcomes.

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Data Availability Statement: The data generated during the current study is not publicly available due to ethical and privacy restrictions associated with human participant data, especially that involves children and vulnerable populations. De-identified data may be made available from the corresponding author upon reasonable request, subject to approval by the relevant Institutional Review Board/Ethics Committee and execution of an appropriate data-sharing agreement.

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Conflicts of Interest: B.Z. and J.L. hold a patent, "Method and device for retinal image analysis," licensed to Health View Bioanalytic Limited, and have received royalties through the Chinese University of Hong Kong. B.Z. and J.L. are founders and shareholders of Health View Bioanalytic Limited. Author Joseph Zee was employed by the company Edtech Bioanalytic Limited. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Abbreviations

The following abbreviations are used in this manuscript:

5-MTHF	5-methyltetrahydrofolate
AI	Artificial intelligence
Aocclusion	Arteriole occlusions
ARIA	Automatic retinal image analysis
AS	Asperger syndrome
ASD	Autism spectrum disorder
ASD + GDD	Co-occurring autism spectrum disorder and global developmental delay
ASD_GDD	Co-occurring ASD and GDD (label used in Tables 1 and 2)
averageC_Dratio	Average cup-to-disc ratio
AverageGCIPL_t or AverageGCIPL_thickness	Average of Ganglion Cell-Inner Plexiform Layer thickness
AVR	Arteriole–venule ratio (i.e., the ratio of CRAE and CRVE)
CAS	Child Assessment Service (Department of Health, Hong Kong)
CDR	Cup-to-Disc Ratio: The ratio of the diameter of the central depressed area of the optic nerve (the "cup") to the total diameter of the optic nerve head (the "disc").
CFD	Cerebral folate deficiency
CI	Confidence interval
CNS	Central nervous system
CRAE	Central retinal artery equivalent
CRSRehab-PS	Central Referral System for Rehabilitation Services—Subsystem for Disabled Pre-schoolers
CRVE	Central retinal vein equivalent
CRVE_L	Central retinal vein equivalent, left eye
CRVE_R	Central retinal vein equivalent, right eye
CSF	Cerebrospinal fluid
CUHK-NTEC	Chinese University of Hong Kong—New Territories East Cluster (Joint Clinical Research Ethics Committee)
Cup_di	Cup Diameter: The absolute diameter measurement of the central optic cup.
Cup_volume	Cup volume(mm^3): A 3-dimensional measurement of the volume of the central depression (cup) of the optic nerve.
Disc_area	Disc area (mm^2): The total area of the optic nerve head

Disc_di	Disc Diameter: The absolute diameter measurement of the entire optic disc.
EETC	Early Education and Training Centre
Exudates	The presence or absence of exudates
FRAA	Folate receptor alpha autoantibody
GDD	Global developmental delay
Grad-CAM	Gradient-weighted class activation mapping
HA	Hospital Authority (Hong Kong)
Hemorrhage	The presence or absence of haemorrhage
HFA	High-functioning autism
IQ	Intelligence quotient
MAangle	Mean of the bifurcation angles of arterioles
MAasym	Mean of the asymmetry index of arterioles (same as MAasymmetry; label used in Tables 1 and 3)
MAasymmetry	Mean of the asymmetry index of arterioles
Mangle	Mean of the bifurcation angles of arterioles (same as MAangle; label used in Tables 1 and 3)
MBCA	Mean of the bifurcation coefficient of arterioles
MBCV	Mean of the bifurcation coefficient of venules
MinGCIPL_t or MinGCIPL_thickness	Minimum of Ganglion Cell-Inner Plexiform Layer thickness
MVangle	Mean of the bifurcation angles of venules
MVasym	Mean of the asymmetry index of venules (same as MVasymmetry; label used in Table 1)
MVasymmetry	Mean of the asymmetry index of venules
Nipping	Arteriole–venous nicking was marked as the narrowing of a venule at the crossing point of an arteriole
OCT	Optical coherence tomography
OPRS	On-site Pre-school Rehabilitation Services
Pre-P1	Pre-Primary One
Radii	Radii refer to radial distances measured from the centre of the optic cup to the specific clock-hour or cardinal-sector margins (Inferior, Superior, Nasal, Temporal).
RDR	Rim-to-Disc Ratio: The ratio of the neuroretina rim width (the healthy nerve tissue between the cup and the disc boundary) to the total diameter of the optic disc.
Rim_area	Rim area (mm^2): The area of the neuroretina rim
Rim_di	The absolute width of the neuroretina rim (often referred to as Rim Width/Rim Area) is particularly focused in specific quadrants.
RNFL	Retinal nerve fibre layer
RNFL_mt or RNFL_mthickness	Retinal Nerve Fibre Layer thickness (μm)
ROC	Receiver operating characteristic
SCCC	Special Child Care Centre
SEN	Special educational needs
SVM	Support vector machine
Tortuosity	Tortuosity assessed by visual grading of one fovea-centred and one disc-centred fundus image from each image
verticalC_Dratio	Vertical cup-to-disc ratio

The suffixes _L and _R denote the left eye and the right eye, respectively (for example, CRVE_L and CRVE_R); other retinal and OCT characteristics are defined similarly.

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