Chapter # 18

INVESTIGATING EARLY SIGNS OF DEVELOPMENTAL DYSLEXIA AT THE PRESCHOOL AGE: THE ROLE OF STRESS AND SYSTEMATIC INTERVENTION

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ABSTRACT
Developmental dyslexia (DD) is a multifactorial, specific learning disorder characterized by dysfunctions of biological, neurophysiological, cognitive, and psychomotor factors. This study investigated the association between the early signs of DD and stress. Variants and methylation levels of genes involved in stress response were studied along with mitochondrial DNA copy number (mtDNAcn), a stress-related indicator. From 306 preschool-age children (5.0–6.0 years) recruited, 10 typically developing and 20 identified 'at risk' of dyslexia were tested. Of the latter group, 10 underwent a systematic intervention program, and the rest constituted the control group. Two screening tests for early identification of DD were administered, while a developmental history and the CBCL 1½–5 form of the ASEBA were completed. Genotyping was performed along with mtDNAcn and methylation levels estimation before and after the intervention. Statistically significant differences were observed within the DD group that underwent intervention on cognitive, psychomotor, and linguistic factors, before and after the intervention. Differences in methylation were observed before and after the intervention, and in mtDNAcn only after the intervention. Stress could be involved in the onset of DD, so early detection may contribute to the implementation of effective interventions, thereby reducing or preventing negative effects in later life.

Keywords: developmental dyslexia, early identification, early intervention, multifactorial phenotype, stress.

1. INTRODUCTION

Developmental dyslexia (DD) is a multifactorial, specific learning disorder characterized by multiple dysfunctions of one or more, biological, neurophysiological, psychomotor, cognitive, and socioemotional factors (Livingston, Siegel, & Ribary, 2018). The onset of symptoms of DD, while systematically recorded and addressed during the school age, becomes apparent from the preschool age onwards (Zakopoulou, Christodoulou, Kyttari, Siafaka, & Christodoulides, 2023). Children 'at risk' of DD meet difficulties in a wide range of domains in a complex framework of interactions, shaping an early DD phenotype, such

as: phonological awareness (Hand, Lonigan, & Puranik, 2022; Kastamoniti, Tsattalios, Christodoulides, & Zakopoulou, 2018), psychomotor development (Treiman et al., 2019; Zakopoulou et al., 2021b), memory, visual and auditory perception, speech perception and selective attention (Kellens, Baeyens, & Ghesquière, 2024; Zakopoulou et al., 2021b), and socio-psycho-emotional development (Jordan, McRorie, & Ewing, 2010).

Several independent studies support that frustration, failure, and difficulties caused by learning difficulties, create a constant fear of failure or real failure, sadness, inadequacy, reduced happiness and self-esteem, stress, anxiety, emotional vulnerability (Exarchou, Simos, Siafaka, & Zakopoulou, 2020; Peterson & Pennington, 2015; Zakopoulou et al., 2019). All these matters can influence an individual's predisposition to DD, regardless of the presence or absence of genetic variations in risk genes (Romeo et al., 2018). In the context of DD etiopathogenesis, stress is considered as important factor (Theodoridou, Christodoulides, Zakopoulou, & Syrrou, 2021), presumably underlying a dysregulation of the Hypothalamus-Pituitary-Adrenal (HPA) axis (Kershner, 2020).

2. BACKGROUND

The HPA axis and the serotonergic system are involved in stress response. The HPA axis is a key element in neuroendocrine stress response and its activation leads to glucocorticoid (cortisol) secretion, which in turn exerts its effects in many biological pathways (Mifsud & Reul, 2018; Stratakis & Chrousos, 1995). Serotonin is a neurotransmitter whose levels influence a variety of behaviors, including the response to stress (Canli & Lesch, 2007; Ehli, Hu, Lengyel-Nelson, Hudziak, & Davies, 2012; Steiger et al., 2007). The serotonin transporter (SERT) regulates serotonin levels and is associated with many neurobiological (mainly psychiatric) stress-associated disorders (e.g. PTSD) and HPA axis dysregulation (Ehli et al., 2012; Iurescia, Seripa, & Rinaldi, 2016). The *FKBP5* gene encodes a co-chaperone of the glucocorticoid receptor, where glucocorticoids bind after their secretion, and participates in the regulation of HPA axis activity (Klengel et al., 2013). A single nucleotide variation (SNV) located in the second intron of the gene, rs1360780, leads to overexpression of the *FKBP5* gene and has been implicated in HPA axis dysregulation (Paquette et al., 2014). The *SLC6A4* gene encodes SERT, the main regulator of the serotonergic system that dynamically influences serotonin action and has been implicated in many neuropsychiatric disorders. The 5-HTTLPR polymorphic region, as well as the rs25531 SNV, inside the gene's promoter region influence SERT expression levels, SERT availability and serotonin reuptake, influencing the organism's response to stress. (Ehli et al., 2012; Iurescia et al., 2016). The gene x environment (GxE) interaction also plays an important role in the emergence of diseases and exposure to stress during early developmental stages can influence the epigenetic profile of an affected individual (Berretz, Wolf, Gunturkun, & Ocklenburg, 2020; Mascheretti et al., 2013; McEwen & Gianaros, 2011; Short et al., 2024). Early life stress exposure can influence the methylation levels of the *SLC6A4* gene and lead to increased methylation of CpGs in the gene's promoter region, both at early and later life stages (Chau et al., 2014; Lesch, 2011; Philibert et al., 2007). Mitochondrial DNA copy number (mtDNAcn) is a sensitive stress biomarker, and increased levels have been associated with maltreatment in children and adults. It has also been associated with internalizing behaviors in children and has been proposed as a marker of abuse and life adversities in preschoolers (Ridout et al., 2019). All these multidimensional difficulties are expected to impact both the child's life and the functioning of the family and/or the school environment (Exarchou et al., 2020; Peterson, 2021; Zuk et al., 2021). As a result, in order to support each individual in the long term, early diagnosis should be a priority for

each family and education system (Colenbrander, Ricketts, & Breadmore, 2018). Early identification of DD focuses on timely and accurate diagnosis, with the goal of preventing later learning and socioemotional difficulties, during the school age.

However, in terms of studying the existence of an early interactive relationship between stress and DD, as well as the importance of this interaction in a multifaceted understanding of the endophenotype and phenotype of DD, a critical literature gap is highlighted.

3. OBJECTIVES

In continuation of our research on the early identification of the complex structure of DD (Zakopoulou et al., 2021a, 2021b; Theodoridou et al., 2021), the purpose of this study is to investigate the early role of stress in this network of strongly interrelated difficulties, through an innovative research protocol.

Aiming to this, we discuss the results of investigating the question whether there is an association between the early signs of DD and stress-related biological factors (HPA axis and serotonergic system genes, mtDNAcn) and if a systematic intervention even from preschool age, can influence these associations.

4. MATERIALS & METHODS

In this chapter we present the results of the analysis investigating the association between early DD signs and the frequency of polymorphisms/variants affecting the expression levels of HPA axis and serotonergic system genes, involved in the regulation of stress response, while also evaluating the mitochondrial DNA copy number (mtDNAcn), a sensitive stress biomarker, in a population of young preschoolers at risk of developing DD and matched controls.

4.1. Data Acquisition

306 Greek preschoolers aged 5.0 to 6.0 years (mean 66.87 months, SD: 4.34) participated in this study, and 8% (24 preschoolers) were found to be ‘at risk’ of DD. All participants were recruited in the wider area of Ioannina and Athens in Greece, through public kindergartens. The study was approved by the Ethics Committee of the Greek ministry of Education (Φ15/121206/ΑΛ/125659/Δ1/13-10-2022). Written consent forms were obtained from all parents and the kindergarten teachers of the children to participate in the initial evaluation. In total, three test groups were formed, as follows: 10 preschoolers identified as ‘at risk of DD with intervention’ were randomly selected to undergo a 3-month systematic intervention program, while 10 did not undergo an intervention program, constituting the control group (‘at risk of DD without intervention’). For comparison, 10 typically developing preschoolers were also involved (see table 1). Co-occurrence with other neurodevelopmental disorders was not recorded in any of the subjects. 20 parents of children ‘at risk’ of DD and 10 parents of typically developing children consented to their child participating in a 3-month intervention program in all assessment and intervention procedures, following initial assessment.

Table 1.
Demographics of the preschool children participating in the study.

Groups	Number of Preschoolers			Mean Age		Parents' Age			
						F		M	
	Male	Female	*N*	Mean	*SD*	*Mean*	*SD*	*Mean*	*SD*
Without DD	3	7	10	66.4	4.01	40.00	5.98	36.60	5.56
At risk of DD with intervention	9	1	10	64.8	3.68	42.40	2.59	36.40	2.59
At risk of DD without intervention	8	2	10	68.5	6.60	42.30	4.50	40.30	4.08
Total	152	154	306	66.87	4.34				

4.1.1. Assessment of Learning Difficulties and Developmental History

Towards early identification of DD, the test of Early Dyslexia Identification (EDIT) (Zakopoulou, 2003) and the ATHINA test (Paraskevopoulos, Kalantzi-Azizi, & Giannitsas, 1999) were administered. Through the EDIT test three sectors (including 8 tasks) were examined, considering: (i) Visual-spatial Abilities (*Sketching, Copying shapes, Visual discrimination / Laterality / Left-right Discrimination*), (ii) Grapho-phonological Awareness (*Phonemes Composition; Phonemes Discrimination; Name Writing*) and (iii) Working Memory (*Phonemes Discrimination, Name Writing, Copying shapes, Visual-verbal Correspondence*). Through the ATHINA test one sector (including 3 tasks) was examined: Short-term Sequence Memory (*Numbers Memory, Pictures Memory, Shapes Memory*). All measurements were administered individually in the kindergarten, without the presence of parents or kindergarten teachers. Seeking to compare indicative early signs of DD with early sings of behavioral stress, a developmental history and the Greek edition of the Child Behavior Checklist for Ages 1½ to 5 (CBCL 1½–5) of the Achenbach System of Empirically Based Assessment (ASEBA) (Roussou, 2009), were implemented, through which internalizing and externalizing problems such as Emotionally Reactive, Anxious, Depressed, Aggressive Behavior, Attention Problems, Somatic Complaints, and Withdrawn, were tested.

4.1.2. Intervention Method

ProAnaGraPho (Zakopoulou & Tsarouha, 2009) is guided to support children between 5-7 years old with early occurrence of neurodevelopmental disorders, including DD. It consists of 79 exercises through which the child is supported to acquire skills from three domains, such as: (a) Visuo-spatial Abilities (six sub-sectors are included: Body Shape, Spatial Orientation, Temporal Sequences, Right-left Discrimination, Ordering, and Visuo-motor coordination); (b) Working Memory (three sub-sectors are included: Visual Working Memory, Audio Working Memory, and Sequence Working Memory); and (c) Grapho-phonological Awareness (two sub-sectors are included: Phonological Awareness and Phoneme-grapheme Correspondence).

Taking into consideration the diagnostic profile of the 10 preschoolers identified as 'at risk of DD with intervention', a series of combined exercises of the ProAnaGraPho method were selected for implementation. The intervention program was implemented in the 2nd semester of the 2nd year of kindergarten in a 3-month individualized intervention program, four times a week.

4.1.3. Genetic, Epigenetic and Mitochondrial DNA Copy Number Analysis

To examine the role of the stress at the molecular level, DNA from buccal cell swabs was extracted from all subjects (all parents were present during the sampling process): (a) mtDNAcn was evaluated by qPCR, using primers to target the nuclear DNA and mtDNA. One sample from the *"at risk of DD with intervention"* group was considered an outlier and removed from the analysis; (b) rs1360780 was genotyped using a TaqMan assay (Thermo Assay ID: C_8852038_10, #4351379, Applied Biosystems, Foster City, CA), while 5-HTTLRP and rs25531 were analyzed using a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay; (c) methylation analysis was performed using pyrosequencing CpG assay with primers that targeted 16 CpG sites in the promoter region of the *SLC6A4* gene.

4.2. Statistics

Multivariate analysis was applied between all variables between the three groups. Data were examined for normality distributions, and non-parametric tests were used in cases where data did not follow Gaussian distribution. Results are presented after correcting for and excluding outliers with the ROUT method. Statistical significance was considered for $p < 0.05$ with 95% confidence intervals (CI).

5. RESULTS

The data provided reflects One-Way ANOVA tests on the changes in developmental skills within each group before (Time 0) and after the intervention (Time 1) (Table 2) and the changes among the three groups of children after the intervention (Time 1) (Table 3), as follows:

(i) Statistically significant differences were observed within the group *'at risk of DD with intervention'* in Time 0 and in Time 1 on specific tasks of the domains of Visual-spatial Abilities (*Sketching, Copying Shapes)*, Grapho-phonological Awareness (*Phonemes Discrimination; Name Writing*), and Working Memory (*Phonemes Discrimination, Name Writing, Copying Shapes*). Within the group *'at risk of DD without intervention'* (Time 0 and Time 1) statistically significant differences were observed on specific tasks of the domains of Visual-spatial Abilities (*Copying Shapes)*, Grapho-phonological Awareness (*Name Writing*), and Working Memory (*Name Writing, Copying Shapes*). No statistically significant differences were observed within the typically developed group (see table 2).

Table 2.
Differences between Time 0 and Time 1 within the groups of children at risk of DD with intervention, at risk of DD without intervention, and children without early signs of DD.

Tested Skills	Groups														
	At risk of DD with intervention					At risk of DD without intervention					Without DD				
	Mean	SD	Median	Range	*p*	Mean	SD	Median	Range	*p*	Mean	SD	Median	Range	*p*
Sk_time 0	2.90	2.51	2.50	6.00	**0,007**	5.10	1.73	6.00	6.00	0,759	8.90	3.41	9.50	12.00	1,000
Sk_time 1	6.80	2.70	8.00	7.00		5.60	3.50	6.50	10.00		8.90	3.41	9.50	12.00	
CSh_time 0	2.80	2.44	2.00	8.00	**0,012**	4.20	1.87	4.00	5.00	**0,020**	7.50	.97	8.00	3.00	1,000
CSh_time 1	6.30	1.34	6.00	4.00		6.50	1.58	6.50	4.00		7.50	.97	8.00	3.00	
VD_time 0	5.70	.48	6.00	1.00	0,317	5.00	2.16	6.00	6.00	0,285	5.90	.32	6.00	1.00	1,000
VD_time 1	5.90	.32	6.00	1.00		5.80	.63	6.00	2.00		5.90	.32	6.00	1.00	
L_time 0	5.20	2.57	6.00	8.00	0,227	5.40	2.72	6.00	7.00	0,753	5.90	2.96	6.00	10.00	1,000
L_time 1	6.00	2.91	5.50	8.00		5.80	3.29	6.00	10.00		5.90	2.96	6.00	10.00	
L-R_time 0	2.50	1.18	2.50	4.00	0,238	2.30	.48	2.00	1.00	0,414	2.00	.82	2.00	3.00	1,000
L-R_time 1	2.50	1.18	2.50	4.00		2.30	.48	2.00	1.00		2.00	.82	2.00	3.00	
NW_time 0	2.80	1.93	3.50	5.00	**0,007**	2.60	1.90	2.50	5.00	**0,005**	7.70	.48	8.00	1.00	1,000
NW_time 1	5.90	1.85	6.00	5.00		5.90	1.60	6.00	5.00		7.70	.48	8.00	1.00	
PhD_time 0	6.50	.71	7.00	2.00	**0,005**	7.00	1.25	7.00	5.00	**0,022**	9.50	.71	10.00	2.00	1,000
PhD_time 1	8.90	.74	9.00	2.00		8.70	.95	9.00	3.00		9.50	.71	10.00	2.00	
V-VC_time 0	1.60	2.32	.00	6.00	0,068	1.20	2.53	.00	6.00	0,157	.00	.00	.00	.00	1,000
V-VC_time 1	.00	.00	.00	.00		.00	.00	.00	.00		.00	.00	.00	.00	

Notes. Sketching=Sk; Copying Shapes=CSh; Visual discrimination=VD; Laterality=L; Left-right discrimination=L-R; Name Writing=NW; Phonemes Discrimination=PhD; Visual-Verbal correspondence=V-VC

(ii) With regard to the changes between the three groups (see table 3), we see that interventions targeting children at risk of DD can lead to significant improvements in the developmental skills of sketching, name writing, and phoneme discrimination. In particular, they recorded substantial improvement with intervention, as their low p-values indicated. However, the intervention did not appear to affect all skills equally, like visual discrimination and laterality, as children performed highly in these even before the intervention, showing no significant change across the groups.

Table 3.
Changes within the groups of children at risk of DD with intervention, at risk of DD without intervention, and children without early signs of DD.

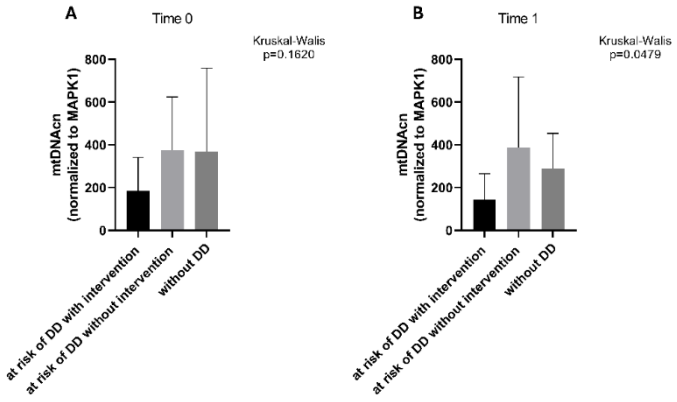
Tested Skills	Groups												*p*
	At risk of DD with intervention				At risk of DD without intervention				Without DD				
	Mean	SD	Median	Range	Mean	SD	Median	Range	Mean	SD	Median	Range	
Sk Change	3.20	3.22	3.00	10.00	.20	2.74	1.00	9.00	.00	.00	.00	.00	**0,011**
CSh Change	2.60	2.84	2.50	9.00	1.50	3.24	.00	9.00	.00	.00	.00	.00	0,081
VD Change	.40	.84	.00	3.00	.80	2.35	.00	8.00	.00	.00	.00	.00	0,472
L Change	2.60	3.27	2.50	11.00	1.80	3.49	1.50	12.00	.00	.00	.00	.00	0,117
L-r Change	.80	2.15	1.50	6.00	.20	1.55	1.00	4.00	.00	.00	.00	.00	0,486
NW Change	2.80	2.20	3.00	6.00	1.70	2.41	1.50	7.00	.00	.00	.00	.00	**0,009**
PhD Change	2.40	1.07	3.00	3.00	1.00	1.25	1.50	3.00	.00	.00	.00	.00	**<0,001**
V-VC Change	-.30	.95	.00	3.00	.00	.00	.00	.00	.00	.00	.00	.00	0,381

Notes. Sketching=Sk; Copying Shapes=CSh; Visual discrimination=VD; Laterality=L; Left-right discrimination=L-R; Name Writing=NW; Phonemes Discrimination=PhD; Visual-Verbal correspondence=V-VC

From the buccal DNA analysis, the following results were recorded:

I. mtDNA copy number: although no statistically significant difference were observed at Time 0 in mtDNAcn between the three groups, at Time 1, a statistically significant difference in mtDNAcn levels was recorded between the 3 groups, (Kruskal Wallis, p=0.048) (see figure 1). The main statistically significant difference recorded was between the *'at risk of DD with intervention'* group and the *'at risk of DD without intervention'* group (Dunn's post-hoc test, p=0.0465). Specifically, as we see in the figure, the measurements are based on groups but also on time. The changes that were observed across time are in all cases non-significant even though the central tendency expressed by the median is indicative of differences.

Figure 1.
mtDNA copy number differences between the three groups at A. the initial (Time 0) and B. final testing state after 3 months (Time 1).



II. Genotyping of variants of stress related genes: Although the "risk" alleles are more common in the dyslexia group, no statistically significant difference is observed (see table 4).

Table 4.
Allelic frequencies of genotyped variants (risk alleles are in bold).

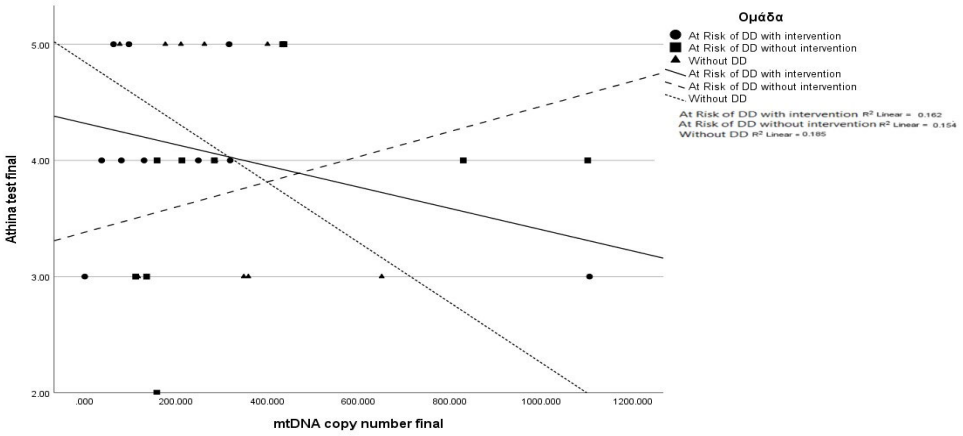
Variant / Polymorphism	Genotype Frequency			Allele Frequency		
	Dyslexia (n=20)	Controls (n=10)	*p*	Dyslexia (n=40)	Controls (n=20)	*p*
FKBP5 rs1360780	CC (8/20 - 40%) C**T** (11/20 - 55%) **TT** (1/20 - 5%)	CC (6/10 - 60%) C**T** (4/10 - 40%) **TT** (-)	*0,6331*	C (27/40-67,5%) **T** (13/40-32,5%)	C (16/20-80%) **T** (4/20-20%)	*0,4783*
SLC6A4 5-HTTLRP	**SS** (5/20 - 25%) S**L** (9/20 - 45%) LL (6/20 - 30%)	**SS** (1/10 - 10%) S**L** (5/10 - 50%) LL (4/10 – 40%)	*0,7751*	**S** (19/40-47,5%) L (21/40-52,5%)	**S** (7/20-35%) L (13/20-65%)	*0,5190*
SLC6A4 rs25531	AA (17/20 - 85%) A**G** (3/20 – 15%) **GG** (-)	AA (9/10 - 90%) A**G** (1/10 - 10%) **GG** (-)	*1*	A (37/40 - 92,5%) **G** (3/40 – 7,5%)	A (19/20 - 95%) **G** (1/20 – 5%)	*1*

III. Furthermore, the effect of several indices was examined on these differences. Specifically, the observed mtDNAcn measurements were examined for differences depending on the presence of internalizing and externalizing problems, according to the answers on CBCL 1½–5. None of these had a statistically significant effect on mtDNAcn values, before or after the intervention or to their change.

IV. A series of correlations were also examined between scores at the EDIT and ATHINA test with the mtDNAcn values and the mtDNAcn observed changes. A statistically significant and positive correlation was observed for the group 'at risk of DD without intervention' highlighting a positive correlation between the persistence of the difficulties and an increase in stress. Regarding the mtDNAcn changes no statistically significant correlations were observed in any group (see figure 2).

Figure 2.

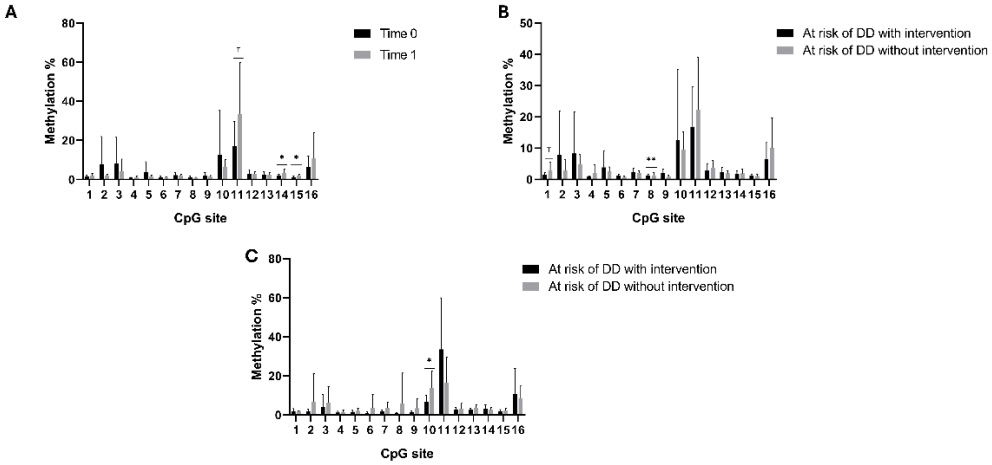
Correlations between ATHINA scores and mtDNAcn values at the final testing stage.



V. The methylation profile of 16 CpG sites in the promoter region of *SLC6A4* was analyzed at Time 0 and Time 1 the 3-month systematic intervention took place. The methylation analysis was performed for the 20 children belonged to the "at risk of DD" group and 3 comparisons were made: 1. methylation levels of the "at risk of DD group" at Time 0 and Time 1, 2. methylation levels of the "at risk of DD group with intervention" and "at risk of DD group without intervention" at Time 0 and 3. methylation levels of the "at risk of DD group with intervention" and "at risk of DD group without intervention" at Time 1 (see figure 3). The first comparison showed a trend (T) for higher average methylation at CpG11 after the intervention (p=0.0874) and a statistically significant increase in CpGs 14 and 15 (p= 0.0156 and p=0.0295, respectively), although it must be noted that the methylation levels in these two CpGs were extremely low both at Time 0 and Time 1. The second comparison showed that a trend (T) for higher average methylation of CpG1 in the "at risk of DD group without intervention" (p=0.0873) and a statistically significant higher methylation in CpG8 (p=0.0074), although it should be highlighted that in both cases the methylation percentage is very low. The third comparison showed a statistically significant lower methylation percentage in CpG10 for the intervention group (p=0.0216), and it should be noted that at Time 0 this CpG showed higher methylation in the "at risk of DD group with intervention" group.

Figure 3.

Methylation percentages of 16 CpG sites in the promoter region of the SLC6A4 gene. A. Comparison between the "at risk of DD with intervention" group before (Time 0) and after (Time 1) the intervention program. B. Comparison between the "at risk of DD with intervention" and the "at risk of DD without intervention" groups at Time 0. C. Comparison between the "at risk of DD with intervention" and the "at risk of DD without intervention" groups at Time 1. Trend (T) indicates a p-value < 0.1, asterisk (*) indicates a p-value < 0.05 and double asterisk (**) indicates a p-value < 0.01.



6. FUTURE RESEARCH DIRECTIONS

The small sample size of the study and lack of sufficient statistical power do not allow us to draw firm conclusions regarding the association between stress and DD. Future studies should implement a prospective design and include a much larger number of participants, have a balanced sex ratio, take into account factors such as various types of dyslexia, interventions, educational and examination systems, psychological support, as well as assessment of the parents along with their children. These studies could provide valuable insights to elucidate the complex interaction network between stress and DD.

7. CONCLUSION/DISCUSSION

The main aim of the current study was to investigate the role of stress in the early DD phenotype.

Contributing to the multifaceted investigation of the early structure of DD, the results of this study, consistent with the findings of other studies (Jordan & Dyer, 2017; Parhiala et al., 2015; Sheehan, 2017; Zakopoulou et al., 2023; Zakopoulou et al., 2021b) emphasize that stress tends to be a component of an 'at risk' for DD clinical entity, without however confirming a cause-effect relationship between them. Specifically, Jordan and Dyer (2017) argued that before school entry, children later diagnosed with dyslexia did not present psychological impairments, but only mild conduct problems, while psychological problems

became more evident when children entered school. Adding to this, the significant correlation observed between stress and the persistence of difficulties in specific domains, such as working memory, rather confirms the Horbach, Mayer, Scharke, Heim, and Günther (2020) statement that internalizing problems increase numerically during the transition from kindergarten to elementary school.

Furthermore, it was found that the children who were diagnosed 'at risk' for DD at the first testing stage, showed a statistically significant improvement in all the domains that initially had recorded low scores, after the implementation of the intervention (Hebert, Kearns, Hayes, Bazis, & Cooper, 2018). As it has been reported (Clark et al., 2014; Fuchs et al., 2012; Lyytinen, Erskine, Hamalainen, Torppa, & Ronimus, 2015), children with DD who attend intervention programs started in preschool and first grade of school, achieve better learning development.

Importantly, the findings underscored that the stress-related risk alleles are more common in children at risk of DD, while mtDNAcn displayed lower levels in the "at risk of DD with intervention" group, without showing statistically significant differences with typically developing children before the intervention. Despite these low levels of mtDNAcn, a reduction in mtDNAcn was observed after the intervention, indicating that early intervention programs contribute positively to minimizing stress levels, confirming relevant research findings (Buchweitz et al., 2023; Smythe, Zuurmond, Tann, Gladstone, & Kuper, 2020; Stein, Hoeft, & Richter, 2024) and highlight the modulatory role of mitochondria in stress-related outcomes (Filiou & Sandi, 2019; Papadopoulou et al., 2019; Thomou et al., 2024). Also, while differences in methylation levels were observed both before and after the intervention between the two groups (at risk of DD with and without intervention), the majority of those statistically significant results concerned CpG sites with very low methylation percentages, making those results uninformative. The only CpG site that had high methylation percentage (>10%) and whose average methylation was significantly different between the two groups was CpG10. Before the intervention this site did not have any significant difference between the two groups, and the "at risk of DD with intervention" group had a higher average methylation than the "at risk of DD without intervention" group. This relationship was reversed after 3 months of the systematic intervention program, indicating that the intervention could have an impact on the epigenetic profile of the participating children, as early childhood is a sensitive period where environmental stimuli are biologically embedded through epigenetic changes (Zhou & Ryan, 2023).

In light of these findings, a stress-related DD phenotype is strongly suggested, indicating the existence of powerful mechanisms that negatively influence the reduction or prevention of multiple later school age difficulties and personality effects in individuals with DD.

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KEY TERMS & DEFINITIONS

Chaperones: proteins that participate in the folding, unfolding and translocation of other proteins.

Co-chaperone: protein that assists in the function of other chaperones.

Cortisol: steroid hormone, produced and released by the adrenal glands, that exerts several effects, including the organism's response to stressful situations.

CpG site: region of DNA where a cytosine nucleotide is followed by a guanine nucleotide. Cytosines in CpG sites can be methylated.

Genotyping: laboratory process through which the presence of certain variants in an individual's DNA is determined.

Glucocorticoid receptor (GR): nuclear receptor protein in which glucocorticoids are bound to exert their actions.

Hypothalamus-Pituitary-Adrenal (HPA) axis: communication system between three organs: the hypothalamus, the pituitary gland and the adrenal glands that acts

Methylation: biological process n in which a small molecule called a methyl group gets added to DNA, proteins, or other molecules. The addition of methyl groups can affect how some molecules act in the body without changing the DNA sequence.

mtDNA copy number (mtDNAcn): measure of the number of mitochondrial genomes per cell that acts as a correlate of mitochondrial function and number.

Single Nucleotide Variant (SNV): a DNA sequence variation of a single nucleotide in a population's DNA sequence, or genome.

ACKNOWLEDGEMENTS

We acknowledge support of this work from the project "Immersive Virtual, Augmented and Mixed Reality Center of Epirus" (MIS 5047221), which is implemented under the Action "Reinforcement of the Research and Innovation Infrastructure," funded by the Operational Program "Competitiveness, Entrepreneurship and Innovation" (NSRF 2014-2020) and co-financed by Greece and the European Union (European Regional Development Fund). In addition, we acknowledge support of this work by the project "Establishment of "capacity building" infrastructures in Biomedical Research (BIOMED-20)" (MIS 5047236) which is implemented under the Action "Reinforcement of the Research and Innovation Infrastructure", funded by the Operational Program "Competitiveness, Entrepreneurship and Innovation" (NSRF 2014-2020) and co-financed by Greece and the European Union (European Regional Development Fund).

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