1 Intravenous immunoglobulin has epigenetic, ribosomal, and immune effects in

2 Paediatric Acute-Onset Neuropsychiatric Syndrome

3

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36

37 Highlights:

38 • Five children with PANS receiving monthly IVIg who had clinical benefit were
39 studied
40 • Three young boys had a regressive (autistic) phenotype, whereas the older
41 two girls had OCD
42 • Single-cell RNA sequencing showed epigenetic, ribosomal, and immune
43 dysregulation in PANS
44 • IVIg treatment had epigenetic and immune-modulating effects in PANS
45 • IVIg altered gene expression of histone methyltransferase and chromatin
46 genes

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Abstract

Paediatric acute-onset neuropsychiatric syndrome (PANS) is characterised by infection-provoked abrupt and dramatic onset of obsessive compulsive disorder (OCD) or eating restriction, along with neurodevelopmental regression. Although the aetiology of PANS remains uncertain, due to a prevailing neuroimmune hypothesis some children receive the immune modulator intravenous immunoglobulin (IVIg). Using single-cell RNA sequencing of peripheral immune cells, we examined gene expression in five children with PANS (mean age 10.2 years, range 5–17, 60% male) who were receiving open-label IVIg. Maternal autoimmunity (n=3) and familial neurodevelopmental/neuropsychiatric disorders (n=5) were present. The index PANS event occurred age 1.8–13 years, characterised by abrupt eating restriction (n=5), developmental regression (n=4), and OCD (n=3).

Single-cell RNA sequencing (144,470 cells) was performed in five patients pre- and post-IVIg, and four controls (mean age 13.5 years, range 11–16, 50% male). In PANS pre-IVIg compared to neurotypical controls, there were downregulated immune pathways (defense response, innate immunity, secretory granules) in most cell types. However, NK cells exhibited upregulated immune pathways (response to corticosteroid), supporting a baseline 'immune dysregulation'. In PANS pre-IVIg compared to controls, ribosomal pathways were upregulated in neutrophils and CD8 T cells, but downregulated in NK cells. Post-IVIg, the previously downregulated immune pathways were upregulated in most cell types, and the baseline ribosomal pathway abnormalities were reversed. Additionally, histone modification pathways (histone methyltransferase, chromatin) were downregulated in neutrophils and NK cells post-IVIg. We propose PANS is an epigenetic immune-brain disorder with cellular epigenetic, ribosomal, and immune dysregulation. Therefore, epigenetic and immune-modulating therapies, such as IVIg, may have a critical role in treating this disabling disorder.

Key words: epigenetics, immune, neurodevelopmental disorders, transcriptomics, RNA sequencing

Introduction

84 Paediatric acute-onset neuropsychiatric syndrome (PANS) is characterised by acute-

85 onset obsessive compulsive disorder (OCD) or eating restriction, plus separation

86 anxiety, developmental regression, and somatic symptoms such as sleep and urinary

87 disturbances(1). PANS is classically triggered by infections, but non-infectious stress

88 triggers have also been reported. PANS is a debilitating condition with significant

89 functional impairments, impacting school performance and social interactions. The

90 behavioural and psychiatric symptoms associated with PANS can be resistant to

91 conventional psychiatric medications and psychological therapies. The exact

92 immune mechanisms driving PANS remain poorly understood, and whether PANS is

93 a discrete entity or part of the neurodevelopmental continuum is unclear(1). We

94 hypothesize that PANS represents a clinical phenotype demonstrating an interplay

95 between environmental and genetic factors, where infection or stress triggers

96 disrupted peripheral-central immune and brain function, resulting in sudden

97 deterioration in behaviour.

98

99 Due to the leading hypothesis that PANS is driven by abnormal immune function,

100 immunotherapy such as steroids, antibiotics, and intravenous immunoglobulin (IVIg)

101 have been used with varying degrees of success(2). IVIg has been reported to be

102 beneficial in some children with severe PANS, improving psychiatric and behavioural

103 symptoms, although randomised controlled trials have yielded mixed results, and

104 controversy regarding the aetiology and therapeutics of PANS remain(3, 4). We

105 previously explored gene regulation through bulk RNA sequencing in children with

106 PANS and unexplained neurodevelopmental regression, which showed an

107 epigenetic, immune, and ribosomal signature(5). Here, we explore the effects of IVIg

108 on peripheral immune cells in PANS through the use of single-cell RNA sequencing.

109

Materials and Methods

Participant selection

112 Children (<18 years of age) met criteria for paediatric acute-onset neuropsychiatric

113 syndrome (PANS) based on 2013 PANS diagnostic criterion(1):

- an abrupt, dramatic onset (defined as within 48 hours) of OCD or severely restricted food intake

- concurrent presence of at least two of the seven categories
  - anxiety
  - emotional lability and/or depression
  - irritability, aggression, and/or oppositional behaviours
  - behavioural regression
  - deterioration in school performance
  - sensory or motor abnormalities
  - somatic signs and symptoms, including sleep disturbances, enuresis or urinary frequency.

125

126 Recruited patients had ongoing debilitating neuropsychiatric symptoms at the time of

127 treatment, despite optimization of conventional psychological or psychiatric

128 treatments. Recruited patients were about to commence for first time (n=2), or were

129 receiving ongoing (n=3) open-label IVIg treatment (1.5-2g/kg per month). In Australia,

130 PANS is currently a Medicare-approved indication for IVIg.

131

Control selection

133   We recruited age- and sex-matched healthy children of hospital workers. The

134   inclusion criterion for controls was absence of neurodevelopmental or

135   neuropsychiatric disorder, autoimmune diseases, and severe allergic conditions.

136

137   *Sample collection*

138   In children with PANS, the baseline blood sample was taken during cannulation, and

139   the post-IVIg blood sample was taken at the end of the IVIg infusion (24 hours after

140   infusion commencement). Both patients and controls had absence of any infections or

141   hospitalizations a month prior to blood taking.

142

143   HIVE™ *Single-cell RNA sequencing, bioinformatic and enrichment analyses*

144   Single-cell RNA sequencing, using the HIVE™ platform, described in Supplementary

145   Material, on five patients with PANS before and after IVIg treatment, as well as four

146   age matched healthy controls.

147

148   RNA sequencing data was analyzed in the R statistical environment with tidyverse,

149   described in Supplementary Material. For HIVE™ *single-cell RNA sequencing,* the

150   Seurat package was used for analysis. Pathway enrichment analysis was performed

151   via Gene Set Enrichment Analysis (GSEA) to obtain Gene Ontology (GO) pathways

152   (FDR <0.05) using the *clusterProfiler* package.

153

154   **Ethics Approval**

155   Ethical approval was granted by the Sydney Children's Hospitals Network Human

156   Research Ethics Committee (HREC/18/SCHN/227, 2021/ETH00356).

157

158 **Results**

159 *Clinical data*

160 Five children (mean age 10.2 (5-17) years, 60% males) diagnosed with PANS, and

161 who were commencing IVIg (2g/kg), or were on regular IVIg treatment (1.5g/kg),

162 were recruited. 3 out of 5 children (the males with autism) had negative trio whole

163 exome sequencing. This is a biological study to explore biological mechanisms of

164 therapy, rather than a study to explore clinical therapeutic effect (case histories in

165 Supplementary table 1 and Supplementary material).

166

167 The age of the index PANS event was 1.8-13 years. Three mothers had

168 autoimmunity (coeliac disease, hypothyroidism, ulcerative colitis), and five probands

169 had a first-degree family history of neurodevelopmental or neuropsychiatric disease

170 (Supplementary table). At first PANS event, four children were typically developing,

171 one had ADHD. Four patients had preceding infections at PANS onset, but no clear

172 trigger in one patient. In the first PANS event, symptoms included abrupt onset

173 eating restriction (n=5), developmental regression (n=4), OCD (n=3), loss of

174 continence (n=4), sleep disturbance (n=2), and emotional dysregulation (n=3). All

175 five had one or more further events, and four have had multiple events, almost all

176 triggered by infection. Abrupt worsening of OCD has been the dominant feature in

177 the subsequent events. Five have received psychological therapies, and four have

178 had conventional medical therapies with some benefit. Convincing clinical benefit of

179 IVIg was reported in 4 of the 5 patients (only partial benefit in other patient), but

180 headache was a common side effect in all patients. All five patients currently carry

181 ongoing emotional and OCD diagnoses, and the three boys have ASD diagnoses

182 (Supplementary material).

183

184   *Single-Cell RNA Sequencing (scRNAseq): UMAP and differentially expressed genes*

185   A total of 144,470 cells were sequenced across 14 samples from five patients pre-

186   and post-IVIg treatment (mean age 10.2, range 5-17 years, 60% males), and four

187   controls, mean age 13.5 (11-16) years, 50% males). Uniform manifold approximation

188   and projection analysis of biological samples revealed 11 distinct cell clusters (Figure

189   1A). Differentially expressed genes (DEGs) with FDR <0.05 of the seven cell types

190   with most DEGs, ranged from 49 to 947 DEGs per cell type in pre-IVIg vs control

191   comparison (Figure 1B, left), with the largest DEGs in neutrophils and B cells. There

192   were 109 to 2347 DEGs per cell type in post vs pre-IVIg comparison, predominantly

193   in CD8 T cells, followed by non-classical monocytes and neutrophils (Figure 1B,

194   right).

195

196   GSEA analysis used a ranked gene list derived enriched GO pathways (FDR <0.05)

197   for individual cell types. We focused on results in neutrophils and NK cells, while the

198   results for other cell types are in Supplementary Materials.

199

200   *Neutrophils*

201   PANS pre-IVIg vs control comparison

202   In PANS pre-IVIg vs control comparison, top five upregulated GO pathways in

203   neutrophils included ribosomal/translation pathways (Figure 1C, in red). Top five

204   downregulated GO pathways included immune pathways such as defense response

205   to symbiont (Figure 1C, In blue).

206

207   PANS post- vs pre-IVIg comparison

208   In PANS post- vs pre-IVIg comparison, top five upregulated GO pathways in

209   neutrophils included immune pathways such as response to bacterium (Figure 1D, in

210   red). We focused on the genes from the 'response to bacterium' pathway. A CNET

211   plot of these genes (Figure 1E) revealed GO biological process subclusters including

212   response to lipopolysaccharide (enriched by *TLR4, IRAK3, MAPK14, MAPKAPK2,*

213   *CXCL8, TNFAIP3*), regulation of cytokine production (enriched by *CD14, JAK2,*

214   *HMGB2, FCGR1A, FCER1G*), and neutrophil chemotaxis (enriched by *S100A8,*

215   *S100A9, S100A12*).

216

217   Top five downregulated GO pathways included ribosomal/translation pathways, RNA

218   processing, and histone modification (Figure 1D, in blue). We focused on the genes

219   from the 'histone modification' pathway. A CNET plot of these genes (Figure 1F)

220   revealed GO cellular component subclusters including histone methyltransferase

221   (enriched by *KMT2B, KMT2C, SETD1B*), histone deacetylase complex (enriched by

222   *HDAC5, JMJD1C*), and chromatin (enriched by *KDM3A, KDM4B, CHD3, EP300,*

223   *KAT6A, GATAD2A, GATAD2B*).

224

225   *Natural Killer (NK) Cells*

226   <u>PANS pre-IVIg vs control comparison</u>

227   In PANS pre-IVIg vs control comparison, top five upregulated GO pathways in NK

228   cells included immune pathways such as response to corticosteroid and myeloid cell

229   activation (Figure 2A, in red). Top five downregulated GO pathways included

230   ribosomal/translation pathways (Figure 2A, In blue).

231

232   <u>PANS post- vs pre-IVIg comparison</u>

233   In PANS post vs pre-IVIg comparison, top five upregulated GO pathways in NK

234   cells included ribosomal/translation, as well as immune pathways such as neutrophil

235   chemotaxis, inflammatory response (Figure 2B, in red). We focused on the genes

236   from the 'cytosolic ribosome' pathway. A CNET plot of these genes (Figure 2C)

237   revealed GO cellular component subclusters large ribosomal subunit (enriched by

238   *RPL genes*), and small ribosomal subunit (enriched by *RPS* genes).

239

240   Top five downregulated GO pathways included cytoskeletal and histone

241   methyltransferase pathways (Figure 2B, in blue). A CNET plot of the genes in

242   'histone methyltransferase' pathway (Figure 2D) revealed enrichment of genes

243   including *KMT2A, KMT5A, SETD2, SETD3, SETD5, ASH1L.* A heatmap of the

244   expression of 11 genes in the 'histone methyltransferase' pathway showed overall

245   upregulation in pre-IVIg PANS vs controls (Figure 2E, in red), followed by

246   downregulation post vs pre-IVIg (Figure 2E, in blue).

247

248   *Enriched pathways across all cell types*

249   The top five upregulated and downregulated GSEA GO pathways of individual cell

250   types in PANS pre-IVIg vs control (Figure 2F, left) and PANS post- vs pre-IVIg

251   (Figure 2F, right) were plotted, showing similarities and differences in pathways

252   across cell types. In the PANS pre-IVIg vs control comparison, there were

253   predominantly downregulated immune pathways such as defense response to virus

254   and secretory granule/vesicle; these pathways were generally upregulated post-IVIg

255   treatment across most cell types. In the PANS pre-IVIg vs control comparison,

256   ribosomal pathways demonstrated diverse expression patterns across cell types,

257   with upregulation in neutrophils and CD8 T cells, but downregulation in NK cells.

258   These ribosomal pathways showed a reversal in direction following IVIg treatment.

259

260   **Discussion**

261   In this single-cell RNA sequencing study of children with PANS and healthy controls,

262   we identified key baseline alterations in epigenetic, ribosomal, and immune

263   pathways in PANS patients, that were reversed after IVIg. In this study, the children

264   with PANS all had debilitating behavioural symptoms and high social and academic

265   impairments. As per previous reports of PANS, there were high rates of

266   neurodevelopmental and neuropsychiatric disorders in family members, and

267   maternal autoimmunity(6). We believe this supports our model of combined genetic

268   (neurodevelopmental and immune), and environmental (maternal autoimmunity

269   during pregnancy) factors, resulting in epigenetic immune-brain dysregulation(7).

270   The PANS phenotype of the three younger males included regression (autistic)

271   features, whereas the two older females had predominant OCD symptoms. This

272   suggests that the clinical presentation of PANS may be linked to gender and age-

273   related vulnerabilities in neurodevelopmental processes.

274

275   This study was not a clinical trial, and PANS patients received open-label IVIg

276   treatment. Previous randomised controlled trials of IVIg in PANS have yielded mixed

277   results(3, 4). One trial which failed to show superiority of IVIg over placebo, involved

278   evaluation 6 weeks after IVIg (3). In our experience, IVIg treatment improved

279   psychiatric and behavioural symptoms, leading to better overall function, but the

280   benefit typically lasted only 2-3 weeks. Patients often suffered further recurrent

281   infection provoked events, and three required prolonged monthly IVIg use. This

282 suggests that the effect of IVIg was not sustained, highlighting the need for further

283 research to understand the baseline abnormality in PANS, in order to identify better

284 or adjunctive therapeutics. Future clinical trials with larger sample sizes, combined

285 with immune, or omics testing, are necessary to clarify the indications for immune

286 treatments and their mechanisms in PANS.

287

288 Firstly, we found that children with PANS exhibited baseline immune dysregulation

289 compared to healthy controls, with predominantly downregulated immune pathways.

290 However, NK cells exhibited upregulated immune pathways at baseline, suggesting

291 that 'immune dysregulation' is a better description than 'immune deficiency' alone.

292 While our study focused on peripheral immune cells, microglia (resident central

293 nervous system immune cells) dysregulation has also been implicated in PANS(8).

294 We hypothesize that an impaired innate immune response, both peripherally and in

295 the central nervous system, increases infection susceptibility, with recurrent

296 infections and chronic inflammation triggering and sustaining symptoms in PANS.

297

298 Following IVIg treatment, the immune pathways that were downregulated at baseline

299 were significantly upregulated compared to baseline. Previous single-cell RNA

300 sequencing studies have also revealed cell-specific mechanistic actions of IVIg in

301 Kawasaki disease, demonstrating that its effects are not uniform but vary according

302 to cell type(9). IVIg has multiple immunomodulatory mechanisms of action, including

303 saturation of Fc receptors, neutralization of idiotypic antibodies, complement

304 inactivation, and cytokine inhibition. We found broad immune-modulating effects of

305 IVIg across all peripheral blood cell types. However, its impact on peripheral-central

306 immune crosstalk and central nervous system cells requires further investigation.

12

307

308 Secondly, we identified ribosomal dysregulation at baseline in children with PANS

309 compared to healthy controls, which was reversed following IVIg treatment. The

310 ribosomal machinery is increasingly recognised to play a role in NDDs(10). During

311 times of cellular stress triggered by environmental factors, ribosome function is

312 altered to conserve energy and promote stress adaptation. Epigenetic machinery,

313 including chromatin remodelling, histone modifications, and stress-responsive

314 transcription factors, play a crucial role in regulating ribosome function during stress

315 by modulating gene expression and translation(11).

316

317 There is increasing support for the role of epigenetics, such as chromatin-related

318 genes, in the pathogenesis of NDDs(12). Monogenic epigenetic disorders involving

319 pathogenic DNA variants in chromatin-related genes such as *KMT2D, CDH7,*

320 *HIST1HIE* have been described in children with infection provoked neuroregression

321 or PANS-like presentations (13). Although children with PANS in this study did not

322 have pathogenic DNA variations in chromatin-related or other epigenetic genes, it is

323 plausible that epigenetic mechanisms play a role in PANS, potentially influenced by

324 common gene variants plus environmental factors. Animal models suggest that

325 maternal inflammatory factors during pregnancy, such as infection-mimics,

326 autoimmune conditions, and obesity, can influence offspring brain and immune

327 function, potentially through epigenetic mechanisms(14). The prenatal inflammatory

328 environment is proposed to prime microglia epigenetically, heightening vulnerability

329 to secondary insults later in life, leading to abnormal behaviour(14, 15). In this study,

330 we observed a high incidence of maternal autoimmunity among our patients,

331 consistent with previous findings in children with PANS (16). Additionally, we

332   identified epigenetic effects, mainly histone and chromatin modifications, of IVIg on

333   peripheral immune cells in PANS. In the NK cell histone methyltransferase activity

334   pathway, histone-related genes, including *KMT, SETD*, and *NSD,* were generally

335   upregulated at baseline in PANS pre-IVIg compared to controls, followed by

336   downregulation after IVIg treatment. The epigenetic effects of IVIg and its

337   downstream impact on immune and ribosomal function should be explored further.

338

339   This study shows the strength of single-cell RNA sequencing technology as a tool to

340   discover biomarkers and explore therapeutic mechanisms of action in small cohorts

341   such as this(17). Limitations of this study include the small sample size and the use

342   of single-cell RNA sequencing on peripheral blood immune cells rather than brain

343   tissue. However, we believe that peripheral blood RNA sequencing will transpire to

344   be a useful biomarker that reflects gene regulation (epigenetics) in NDDs (5). Further

345   functional immune testing is essential to confirm and better characterize the immune

346   dysregulation that we identified through RNA sequencing. Although we identified

347   changes in the expression of epigenetic genes, further targeted epigenetic analyses

348   such as histone modification profiling, ATAC sequencing, or methylation analysis, will

349   be essential to further explore the role of epigenetics in PANS and the effects of IVIg.

350   Additionally, given the transient effect of IVIG observed in these patients, identifying

351   alternative oral or dietary treatments with similar immune and epigenetic

352   mechanisms of action could be useful.

353

354   **Conclusion**

355   In five children with severe PANS who clinically responded to IVIg, we identified

356   baseline abnormalities in epigenetic and ribosomal regulation, along with

14

357   downstream immune dysfunction in PANS, and demonstrate that IVIg exerts both

358   epigenetic and immune effects.

359

360   **Data Availability**

361   Anonymized data not published within this article will be made available by request

362   from any qualified investigator.

363

364   **Conflict of interest**

365   There are no competing interests to declare.

366

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373

374   **Author contributions**

375   VH: Data curation, Formal analysis, Investigation, Methodology, Roles/Writing- original

376   draft; HN: Data curation, Formal analysis, Investigation, Roles/Writing- review &

377   editing; BK: Data curation, Formal analysis, Investigation, Roles/Writing- review &

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380   editing; RD: Formal analysis, Investigation, Methodology, Roles/Writing- review &

381   editing; JH: Data curation, Formal analysis, Investigation, Roles/Writing- review &

382 editing; SM: Data curation, Formal analysis, Investigation, Roles/Writing- review &

383 editing; SP: Conceptualization, Data Curation, Investigation, Methodology,

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386 Writing- review & editing

387 SP and RD share responsibilities as senior authors.

388

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407 **Legend**

408 **Figure 1: Single cell-RNA sequencing in children with PANS pre and post IVIg**

409 (A) Uniform manifold approximation and projection (UMAP) analysis identified 11

410 distinct cell clusters

411 (B) Bar chart of differentially expressed genes (DEGs) across 7 cell types in PANS

412 pre-IVIg vs control (left), and PANS post- vs pre-IVIg (right), showing the number of

413 upregulated (red) or downregulated (blue) DEGs.

414 (C) **Neutrophils- PANS pre-IVIg vs control comparison**: Bar plot of the top 5

415 upregulated and downregulated GSEA GO pathways

416 (D) **Neutrophils- PANS post- vs pre-IVIg comparison:** Bar plot of the top 5

417 upregulated and downregulated GSEA GO pathways

418 (E) Connectivity network enrichment plot of the upregulated 'response to bacterium'

419 GO pathway in Neutrophils (PANS post- vs pre-IVIg comparison). Dot size

420 corresponds to number of genes enriching that pathway.

421 (F) Connectivity network enrichment plot of the downregulated 'histone modification

422 GO pathway in neutrophils (PANS post- vs pre-IVIg comparison). Dot size

423 corresponds to number of genes enriching that pathway.

424

425 **Figure 2: Single cell-RNA sequencing in children with PANS pre and post IVIg**

426 (A) **NK cells- PANS pre-IVIg vs control comparison**: Bar plot of top 5 upregulated

427 and downregulated GSEA GO pathways.

428 (B) **NK cells- PANS post- vs pre-IVIg comparison:** Bar plot of top 5 upregulated

429 and downregulated GSEA GO pathways.

17

430   (C) Connectivity network enrichment plot of the upregulated 'cytosolic ribosome' GO

431   pathway in NK cells (PANS post- vs pre-IVIg) comparison. Dot size corresponds to

432   number of genes enriching that pathway.

433   (D) Connectivity network enrichment plot of the 'histone methyltransferase' GO

434   pathway in NK cells (PANS post- vs pre-IVIg) comparison. Dot size corresponds to

435   number of genes enriching that pathway.

436   (E) Heatmap of genes in 'histone methyltransferase' GO pathway by average log2

437   fold change, with pre-IVIg vs control (left) and post- vs pre-IVIg comparison (right).

438   (F) Dot plot visualising the top 5 upregulated and downregulated GSEA GO

439   pathways across cell types in PANS pre-IVIg vs controls (left) and PANS post- vs

440   pre-IVIg (right). Significant pathways (FDR <0.05) were simplified, and only those

441   that were present in > 3 cell types in PANS pre-IVIg vs control comparison were

442   plotted. Dot size represents the -log10(padj) of the pathway, while the colour

443   intensity represents the normalized enrichment score (NES).

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