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Identification of a different DNA methylation pattern for pain related genes in children with intellectual disability: a cross-sectional study

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Abstract

Background Children with intellectual disability are more likely to experience chronic and neuropathic pain, which remains frequently under-recognized due to limitations in self-reporting and objective assessment tools. Epigenetic mechanisms, particularly DNA methylation, are believed to influence pain perception. This study investigates the differences in methylation patterns between children with intellectual disability and their age- and sex-matched neurotypical controls.

Methods A cross-sectional study was carried out from March 2022 to July 2023 at the IRCCS Burlo Garofolo in Trieste, Italy. The methylation profiles of children with severe to profound intellectual disabilities and a history of recurrent pain episodes were compared to those of healthy controls. Whole blood DNA was analysed through bisulfite conversion and microarray technology, focusing on CpG islands, gene regions, and enhancers.

Results Seventy-two participants were enrolled, including 36 children with intellectual disability and 36 without. The analysis identified four Differentially Methylated Regions (DMRs). DMR1, located within the *RNF39* gene, was found to be hypomethylated in children with intellectual disabilities. Similarly, DMR2, upstream of *VTRNA2-1* and *MIR886*, and DMR4, at the 5'UTR of *AURKC*, were also hypomethylated. In contrast, DMR3, positioned upstream of *IRF6*, was hypermethylated in the intellectual disability group.

Conclusions These findings indicate that children with intellectual disability exhibit distinct methylation patterns of genes related to pain pathways compared to their healthy peers. Additional research is needed to investigate the clinical relevance of these epigenetic alterations and their potential as biomarkers for pain in this population.

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Key message

Pain in children with intellectual disability often evolves in neuropathic pain, different epigenetic profiles in pain-related genes could explain their unique pain evolution. Recognizing these differences may support the development of specific pain management strategies.

Keywords Severe neurological impairment, Methylation, Synaptogenesis

Introduction

Severe Neurological Impairment (SNI) refers to a collection of central nervous system disorders occurring in childhood, leading to motor and cognitive impairment as well as medical complexities, necessitating significant support for daily activities [1]. The impairment is typically permanent, with potential for either progressive or static manifestations. Communication with patients with SNI pose significant challenges, as deciphering their emotional responses to daily activities can be complex, even for caregivers [2–6]. In this context, an important issue is pain perception considering this population is prone to pain experience and its under-recognition [7–9]. Risk factors mainly include the presence of multiple morbidities, the underreporting of pain, challenges in communication and the observation of self-injurious behaviour [3, 7, 10–13]. Since these patients typically experience recurrent or chronic pain over the years, along with a significant amount of iatrogenic pain (surgery, painful procedure, devices), a self-perpetuating sensitization mechanism may occur, often ending in neuropathic or nociceptive pain [14–16] (Fig. 1). Studies on chronic pain in the general population highlighted the importance of epigenetic modifications in shaping the perception and processing of nociceptive signals. DNA methylation has been adopted in regulating genes associated with pain transduction, neurotransmission, and immune responses [17–19]. Methylation changes in genes involved in pain pathways have been observed in animal models and human chronic pain patients, indicating the potential role of epigenetic regulation in pain sensitization and chronicity. In the context of children with SNI, the relationship between DNA methylation and pain perception is an emerging area of interest. It is conceivable that alterations in DNA methylation patterns might contribute to the evolution of the pain history of children with SNI [20]. Epigenetic modifications could impact nociception, pain perception, and neural circuitry genes, influencing how these children perceive and respond to painful stimuli [21]. Moreover, since genetic mutations or copy number variations can cause ID, investigating the interplay between genetic and epigenetic factors is essential for comprehending the intricate mechanisms governing pain in this population [17]. Although limited, existing research began to shed light on the potential involvement of DNA methylation in the pain experiences of children with SNI [22]. All of these studies, however, assess methylation patterns in nucleated blood cells under the assumption that these profiles are representative of those found in distinct brain cell populations. This assumption is supported by evidence from genome-wide methylation analyses [23], as well as by a disease-specific study in the context of neurodegeneration [24, 25]. Epigenetic studies focusing on pain-related genes in SNI population may provide insights into the molecular basis of altered pain perception and aid in identifying potential biomarkers for pain assessment, suggesting a rationale for future possible pharmacologic and non-pharmacologic interventions.

This study aimed to explore the presence of distinct methylation patterns, with a particular focus on pain related genes, in pediatric subjects with and without SNI.

Materials and methods

We performed a cross-sectional study between March 2022 and July 2023 at the tertiary-level university teaching children's hospital Institute for Maternal and Child Health IRCCS "Burlo Garofolo" in Trieste, Italy.

Subjects

Children aged 1 to 17 years, with or without severe to profound ID, scheduled for routine blood tests, were consecutively enrolled. SNI was defined according to DSM V criteria as a disorder with onset during the developmental period, characterized by deficits in both intellectual and adaptive functioning across conceptual, social, and practical domains. Based on this definition, four levels of severity were identified: mild, where individuals can live independently with minimal support; moderate, where independent living may be possible with moderate support, such as in group homes; severe, requiring daily assistance with self-care and safety supervision; and profound, necessitating 24-hour care. For the purposes of this study, severe and profound levels were grouped together as a single category. In this regard, an additional inclusion criterion was a classification at level IV or V on the Gross Motor Function Classification System (GMFCS). Pain history both in patients group and control group was recorded by mean of a structured checklist in terms of previous diagnosis of painful conditions needing treatment (e.g. gastro-oesophageal reflux, constipation, hip subluxation, contractures, bone fractures, tooth decay, renal stones, neuropathic pain) according to

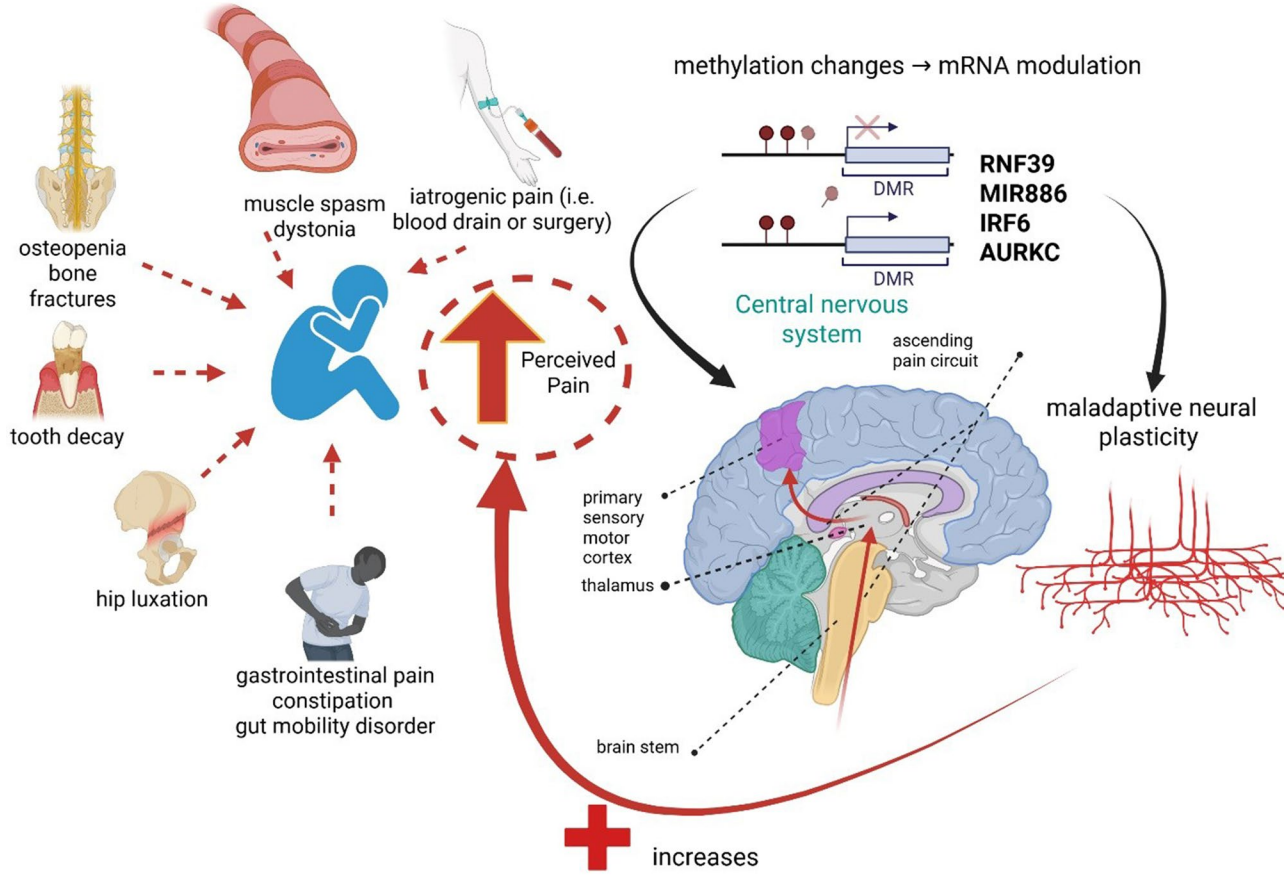

Fig. 1 Chronic pain, stemming from various etiologies, induces alterations in gene methylation levels. This process is associated with heightened neuronal plasticity which, rather than mitigating, exacerbates nociception. *Created in BioRender. Zupin, L. (2025)*https://BioRender.com/ei4jpcc

age. Painful conditions were listed according to recent literature about common pain sources in children with ID. We recorded also surgical and medical procedures, blood samplings and the number of hospitalizations. For the healthy control group, out-patients scheduled for routine or follow-up blood tests were assessed. Participants were excluded if they had a history of chronic medical or psychological conditions, recurrent or chronic pain, or frequent hospitalizations. Chronic conditions were defined according to the International Statistical Classification of Diseases and Related Health Problems-10th Revision. Chronic pain was defined according to the temporal criteria of International Association for the Study of Pain (IASP) with pain lasting more than 3 months. A dedicated researcher carefully reviewed the clinical records of each patient.

Patients and controls were matched by age and sex 1:1 with no further stratification over the same study period. Patients with SNI were categorized as 'P' (i.e. patients) and healthy controls as 'H' (i.e. Healthy).

DNA isolation and bisulfite reaction

Blood was collected from each subject using two standard 4 ml EDTA tubes and stored at -80 °C until further use. Genomic DNA from patients and controls was extracted from 1 ml of blood using a salting-out extraction method according to the method published by Miller et al. 1988 [23] and stored at -20 °C until analysed. Quality control performed with a UV spectrophotometer (N60, NanoPhotometer Implen, Munich, Germany) was repeated, before storage and extraction, when the 260/280 ratio fell outside the 1.8–1.9 range and 260/230 ratio deviated from 2.0 to 2.2 values. The day before the DNA methylation analysis, 500 ng of DNA was subjected to bisulfite conversion, performed using the Zymo EZ96 DNA Methylation Kit (Zymo, catalogue D5003) according to the manufacturer's instructions.

DNA methylation analysis

We utilized the Infinium HD Methylation Assay Protocol (Illumina, San Diego, CA, USA) that scales methylation profiling of thousands of CpG loci per sample, resulting in the interrogation of over 850,000 methylation sites quantitatively across the genome at single-nucleotide

resolution. We prepared DNA for the Infinium MethylationEPIC BeadChips kit according to the Illumina Infinium HD Assay Methylation Protocol Guide (available at https://support.illumina.com/downloads/infinium-hd-methylation-reference-guide-15019519.html). Bisulfite-converted DNA underwent amplification, fragmentation, precipitation, DNA resuspension, and hybridization to the BeadChip. Finally, BeadChips were imaged on an Illumina HiScan system.

DNA methylation analysis

"ChAMP" (Chip Analysis Methylation Pipeline) package for R software (version 4.3.1, R core Team R: A Language and Environment for Statistical Computing 2023) was used to analyze raw data from Illumina Infinium HD Methylation assay [24, 25]. Raw data were loaded into the script and filtered according to the method: 937,055 probes (methylated and unmethylated) were loaded, 3868 probes with a detection p-value above 0.01, 14,119 probes with beadcount < 3 in at least 5% of samples, 31,266 probes overlapped with SNPs, 3630 probes with no CpGs, 21,574 probes located on X, Y chromosome, were eliminated. The resulting 862,598 probes were quality-checked and imputed on the missing values using a custom script. We normalized the type I and II probes and repeated the quality check.

A "Combat" algorithm was employed to assess the batch effect (i.e. a tendency of data to cluster on a specific covariate). Principal component analysis (PCA) evidenced a strong effect in "Slide", considering the other covariates as "Array", "Slide", "Sample Well", "Age", "Sex", "Sample Group". After applying the "Combat" algorithm, PCA indicated only a small batch effect ($p \leq 0.05$) in "Array" and "Sample group". Subsequently, we analysed corrected beta values with a linear model to assess differentially methylated probes between P and H subjects. We used the "Bumphunter" algorithm to analyze and detect any differentially methylated DNA regions [26, 27]. Differentially Methylated Regions (DMR) were defined as genetic contiguous regions containing different CpG island that present similar methylation levels.

Gene Set Enrichment Analysis was performed using "ChAMP" package, using pathway information downloaded from Molecular Signatures Database (MSigDB) and analyzed using a Bayesian algorithm that use a global test to directly find significant genes, with the bias of inequality of CpG number corrected for each gene.

Table 1 Population characteristics

	N	H, *N* = 36[1]	P, *N* = 36[1]	*p*-value[2]
Sex	72			> 0.9
F		15 (42%)	15 (42%)	
M		21 (58%)	21 (58%)	
Age mean (IQR)		9.8 (5-13.5)	10.6 (7–14)	
Diagnosis	72			< 0.001
UN		36 (100%)	0 (0%)	
infantile cerebral palsy		0 (0%)	16 (44%)	
epilepsy		0 (0%)	8 (22%)	
genetic syndrome		0 (0%)	12 (33%)	

[1]n (%)[2]Fisher's exact test

F/M: Female/Male. Diagnosis: grouped population for four classes: UN, Unrelated diagnosis, without ID; Infantile cerebral palsy, patients suffering from perinatal cerebral palsy; Epilepsy, presence of seizures, either tractable or intractable, of different causes; Genetic syndrome, hereditary condition due to defect in one or more genes causing SNI

Statistical analysis

Using the "Bumphunter" algorithm, a statistically significant threshold was fixed at $p < 0.001$, due to the indication in the original paper, suggesting that a higher threshold can decrease the sensitivity of the system [26, 27]. Statistical analysis was performed using the ChAMP package for R software.

No similar studies were available in the literature to define a sample size. No formal power analysis was conducted prior to the study to determine the optimal sample size. The sample size of 72 participants was based on feasibility and the available population during the study period. This may have affected the statistical power to detect smaller effect sizes in DNA methylation differences. However, despite this limitation, we were able to identify four statistically significant DMR with $p < 0.001$, suggesting that the observed differences represent robust epigenetic alterations. Future studies with larger sample sizes, determined through appropriate power calculations, would be valuable to confirm our findings and potentially identify additional methylation differences.

Ethics

The Independent Committee for Bioethics approved the research, following the Declaration of the World Medical Association (registration number IRB-BURLO 01/2022 09.02.2022). Informed consent was previously obtained from parents after full explanation regarding the study.

Results

Seventy-two individuals were enrolled, 36 with severe SNI (P) and 36 without SNI (H), respectively. Table 1 shows the baseline characteristics of patients enrolled in the study; the two groups were similar in terms of demographic characteristics such as sex and age. Distribution of diagnosis of patients with SNI is also indicated in Table 1.

Supplementary Table S1 describes incidence of the main causes of pain according to age in the two groups. Supplementary Table S2 details number of blood test and hospitalization per year in the two groups.

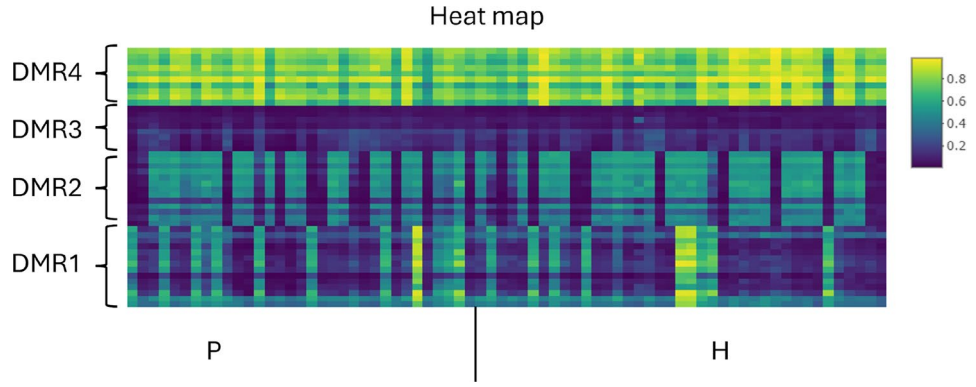

Fig. 2 Heatmap of all the differential methylated regions (DMR) with a statistically significant difference between Patients (P) and Healthy peers (H). In ordinate, each row represents a single probe pertaining to the indicated DMR, while in abscissa each column represents a single individual belonging to either P or H group

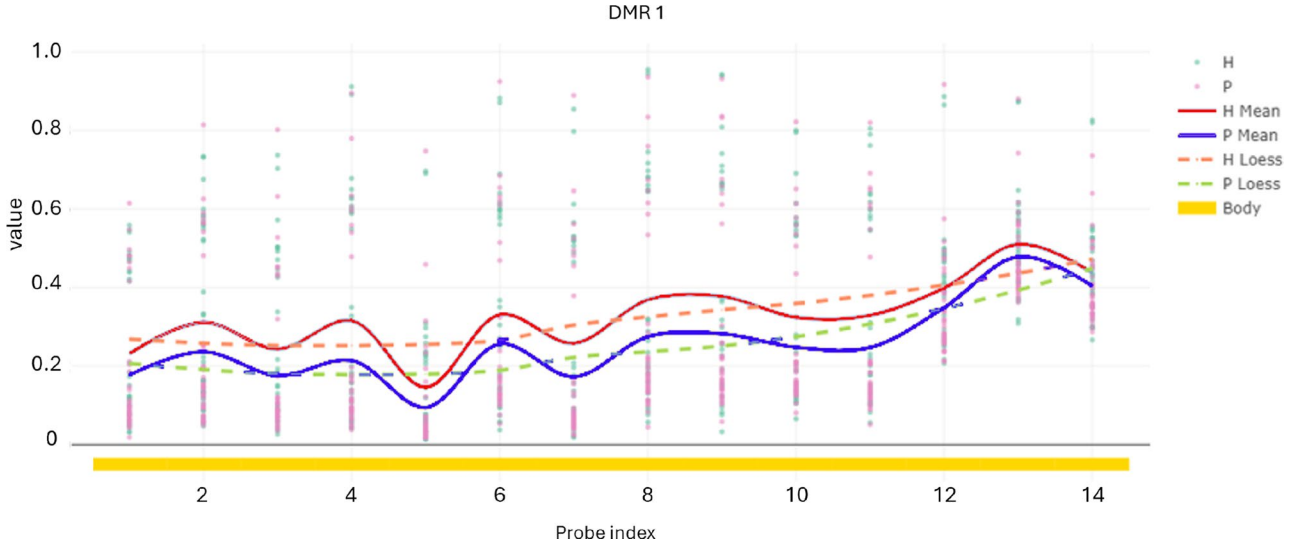

Fig. 3 Differential Methylated Region 1 (DMR 1). The scheme depicts the probes pertaining to this region (probe index) and their specific b-index (value). Each point indicates the β-index for a single individual, either Healthy control (H) or Patient (P) (green and pink points, respectively). Solid lines show the mean trend for β-index, blue for H and yellow for P, while dashed lines show the β-index trend calculated using a "loess" algorithm. Body indicates the position of the probes inside the gene structure, i.e. the body of the gene. DMR 1 encompasses a region from position 30,071,364 to position 30,072,023 in chromosome 6, spanning exon 4 and intron 3 of RNF39 gene

Differentially methylated probes

No probe had a specific statistically significant difference between P and H, indicating that no single CpG has a change in methylation level reaching statistical significance.

Differentially methylated regions

We identified four distinct Differentially Methylated Regions (DMR) with statistically significant changes in methylation levels, represented in Fig. 2 as a heat map. As indicated in "Materials and Methods" section, DMR consists of contiguous regions of CpG island that possess similar methylation levels. There was no visible "batch effect", i.e. areas (either on CpGs index on abscise axis or samples index on ordinate axis) with striking changes in methylation levels. Using the "Bumphunter" algorithm allowed us to eliminate apparent changes in methylation levels due to technical differences.

DMR 1 encompassed an area of approximately 700 bp, marked by 14 different probes; a statistically significant difference between P and H was observed, with P showing a lower methylation level in all the probes included in DMR 1 (Fig. 3). This differentially methylated region encompassed the body of the gene known as *RNF39*, specifically the last coding exon.

DMR 2 was an area of approximately 900 bp, identified by 13 different probes upstream *VTRNA2-1 MIR886*. A statistically significant difference between patients and healthy individuals was observed, with P with a

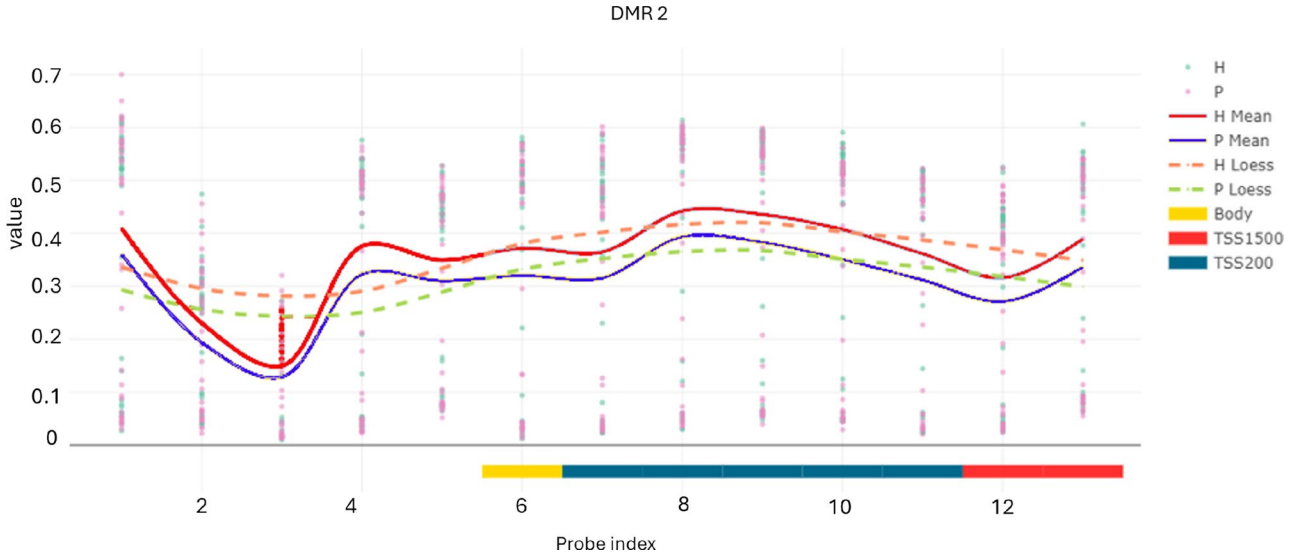

Fig. 4 Differential Methylated Region 2 (DMR 2). The scheme depicts the probes pertaining to this region (probe index) and their specific b-index (value). Each point indicates the β-index for a single individual, either Healthy control (H) or Patient (P) (green and pink points, respectively). Solid lines show the mean trend for β-index, blue for H and yellow for P, while dashed lines show the β-index trend calculated using a "loess" algorithm. Body indicates the position of the probes inside the gene structure, i.e. the body of the gene, while TSS1500 and TSS200 indicate a Transcription Start Site situated 1500 bp and 200 bp respectively from transcription. DMR 2 encompasses a region from position 136,080,003 to position 136,080,923 in chromosome 5, spanning exon 1 and untranslated region of gene MIR886

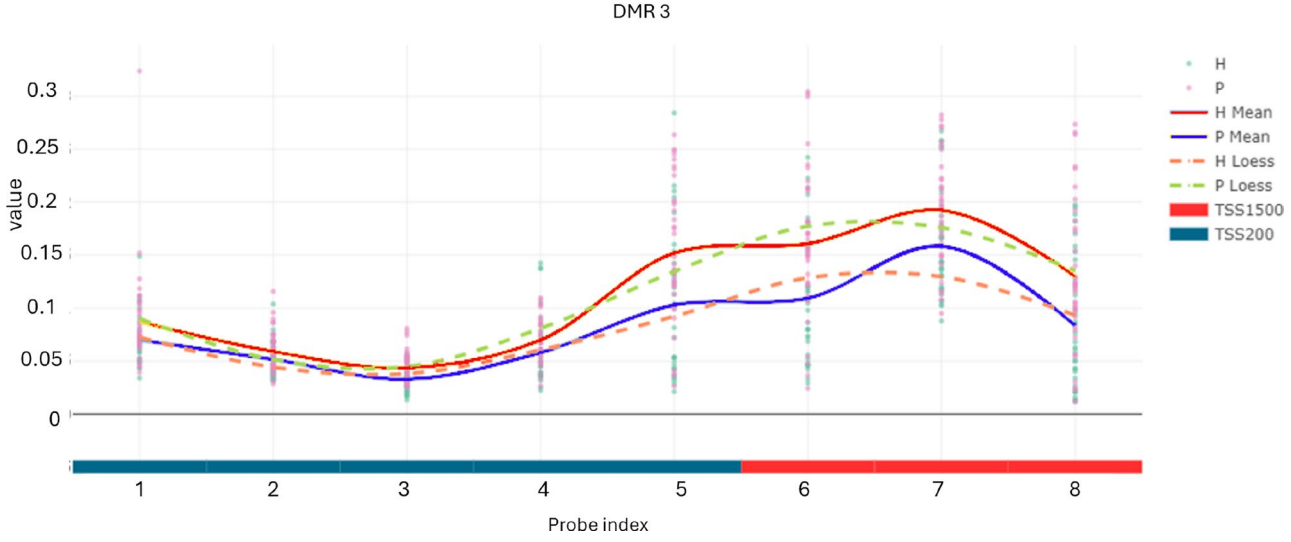

Fig. 5 Differential methylated region 3 (DMR 3). The scheme depicts the probes pertaining to this region (probe index) and their specific β-index (value). Each point indicates the β-index for a single individual, either Healthy control (H) or Patient (P) (green and pink points, respectively). Solid lines show the mean trend for β-index, blue for H and yellow for P, while dashed lines show the β-index trend calculated using a "loess" algorithm. TSS1500 and TSS200 indicate a Transcription Start Site situated 1500 bp and 200 bp respectively from transcription. DMR 3 encompasses a region from position 209,806,141 to position 209,806,433 in chromosome 1, spanning the transcription start site

consistently lower methylation level in all the probes encompassing DMR 2 (Fig. 4).

DMR 3 spanned approximately 300 bp, determined by 8 different probes; the P group displayed increased methylation levels across all the probes identified, but higher in the region of TS1500, upstream of Interferon Regulatory Factor 6 (*IRF6)* gene (Fig. 5). This gene produced a protein belonging to the interferon regulatory transcription factor (IRF) family.

DMR 4 lengthened approximately 300 bp and contained 10 different probes; it was composed of TSS1500, TSS200 and 5'UTR of the *AURKC* gene, and it was hypomethylated in P. Aurora Kinase C (*AURKC*) gene produced a protein that belonged to the Aurora subfamily of serine/threonine protein kinases (Fig. 6).

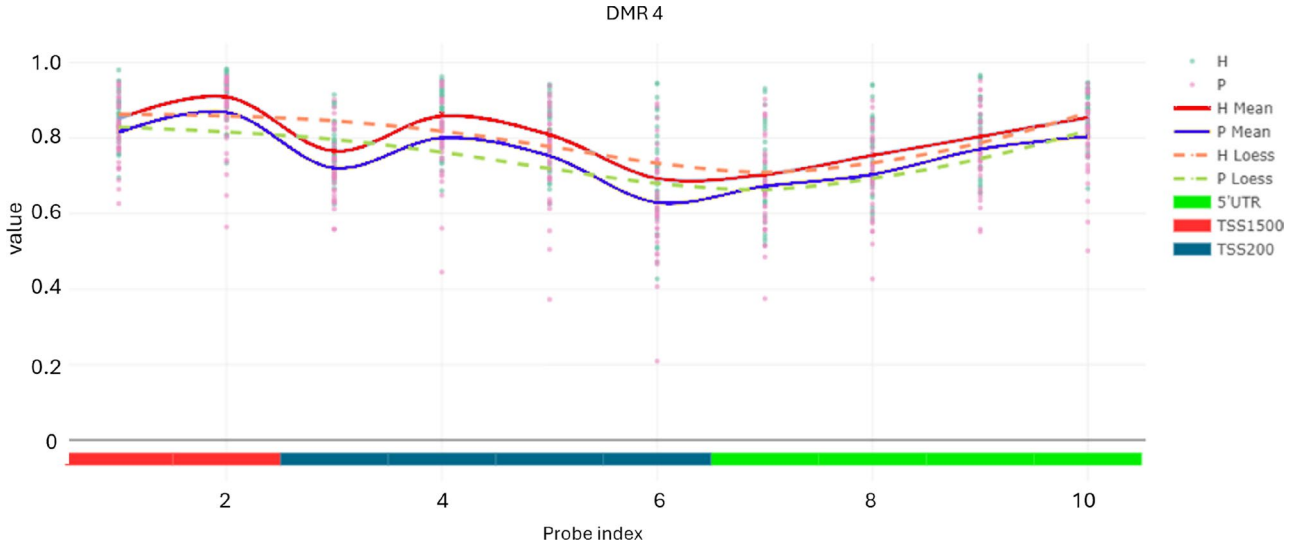

Fig. 6 Differential Methylated Region 4 (DMR 4). The scheme depicts the probes pertaining to this region (probe index) and their specific β-index (value). Each point indicates the β-index for a single individual, either Healthy control (H) or Patient (P) (green and pink points, respectively). Solid lines show the mean trend for β-index, blue for H and yellow for P, while dashed lines show the β-index trend calculated using a "loess" algorithm. TSS1500 and TSS200 indicate a Transcription Start Site situated 1500 bp and 200 bp respectively from transcription, 5'UTR maps the UnTranslated Region at 5'. DMR 4 encompasses a region from position 57,230,743 to position 57,231,075 in chromosome 19, spanning transcription start sites and 5'UTR

Table 2 Summary of DMR characteristics

DMR *N°*	Gen. Location	Gene name	methylation	Δβ	*p*-value
1	chr 6:30071364–30,072,023	RNF39	↓	-0.701	$8.244x\text{-}10^{-6}$
2	chr 5: 136,080,003–136,080,923	MIR886	↓	-0.435	2.778×10^{-3}
3	chr 1: 209,806,141–209,806,433	IRF6	↑	0.466	1.237×10^{-4}
4	chr 19: 57,230,743–57,231,075	AURKC	↓	-0.211	1.319×10^{-4}

DMR N°: progressive number of DMR. Gen. Location: genetic coordinates for the DMR. Gene name: Gene symbol for the area comprised in the DMRs. Methylation: indication of direction of change in methylation levels in P compared to H. Db: b value changes between H and P in DMR area. p-value: corrected p-value for the DMR area

Table 2 summarizes the principal characteristics of DMR detected in this study.

Discussion

This study reveals distinct DNA methylation patterns in pain-related genes between children with SNI and age-matched controls without SNI, suggesting a role for epigenetic regulation in this population. Specifically, we identified four DMRs between groups.

DMR1 encompassed the RNF39 gene and was hypomethylated in children with SNI. RNF39 encodes RING Finger Protein 39, previously linked to long-term potentiation in hippocampal cells [28]. DMR2 was found upstream of VTRNA2-1/MIR886, also hypomethylated in SNI patients. This non-coding RNA has been associated with modulation of neuronal differentiation and function [29–30]. DMR3, upstream of IRF6, was hypermethylated in SNI patients. IRF6 encodes a transcriptional regulator involved in neural tube and cranial neural crest development [31]. Finally, DMR4 was located upstream of AURKC and showed decreased methylation in SNI patients. AURKC encodes a chromosomal passenger protein involved in microtubule organization and has been implicated in neuronal morphogenesis, specifically in neurite outgrowth and dendritic arborization [32]. Gene set enrichment analysis confirmed that synaptogenesis was the principal pathway affected by differential methylation, consistent with the notion that SNI involves widespread synaptic dysfunction.

The hypomethylation of *RNF39* may relate to impaired memory regulation and altered pain perception, consistent with the clinical picture of children with SNI—who often face chronic pain, repeated hospitalizations, and stressful medical procedures. Additionally, a lack of cortical defensive mechanisms may amplify stress responses, potentially creating a self-reinforcing cycle of distress. Similarly, upregulation of *VTRNA2-1* may disrupt synaptogenesis and structural plasticity. Since synaptic activity drives dendritic arborization, any early alteration could impact neural connectivity and processing, especially in a context of brain injury or neurodegeneration. The hypermethylation of *IRF6* suggests impaired neural development. While some in vitro models show protective effects of *IRF6* silencing after trauma [33], these findings may

not directly translate to the complex epigenetic landscape observed in SNI.

Previous research supports a link between altered DNA methylation and pain-related conditions. For example, individuals with fibromyalgia or chronic fatigue syndrome exhibit methylation changes in genes tied to immune and cellular signalling pathways [34]. In fibromyalgia, increased methylation of the *GRM2* gene hints at glutamatergic involvement in chronic pain [17]. Moreover, methylation of genes associated with depression and inflammation further supports an epigenetic contribution to disease manifestation. Contrasting findings emerge from a previous study of individuals with SNI that failed to detect significant methylation signatures—likely due to methodological or sampling differences [22]. However, other pediatric studies show enrichment of neural regulatory pathways linked to chronic postsurgical pain and anxiety sensitivity through differential DNA methylation [35]. Animal studies also corroborate this link; repetitive neonatal pain increased methylation of the *Mor-1* promoter in rats' spinal cords, emphasizing early life experiences' long-term epigenetic impact [36].

We hypothesize that the observed methylation changes may be driven by chronic exposure to pain and stress—common in children with SNI—combined with impaired cognitive and emotional regulation. These changes might reflect maladaptive neural plasticity in response to persistent nociceptive input, exacerbated by limited cortical modulation. Understanding these epigenetic alterations could inform future strategies for early intervention and pain management tailored to neurodevelopmentally vulnerable populations.

Limitations

Our study has several limitations. Firstly, it was no possible to identify specific CpG island methylation change linked to SNI; it is probable due to both the limited number of sample and the heterogeneity of the conditions included in the definition of SNI. Secondly, due to its cross-sectional design, we are unable to draw conclusions about causality between the identified DMR, and the clinical characteristics observed. The temporal sequence of events cannot be established, limiting causal inference. Additionally, we could not definitively determine whether the identified DMRs were associated with the specific underlying disease or rather with recurring and disrupted pain perception. We did not investigate methylation patterns for each individual condition with a specific genetic diagnosis, and our findings encompassed a range of clinical conditions, from neurodegenerative diseases to hypoxic brain injuries. This heterogeneity in our sample may limit the generalizability of our conclusions. However, it also serves as a strength, as the various diagnoses collectively align with the DSM-5 criteria for severe intellectual disability. We chose to emphasize the history of exposure to painful or potentially painful experiences over specific diagnoses, which may not necessarily contribute to understanding this context. Secondly, methylation patterns in blood cells could not necessarily mirror those in the brain. However, this limitation is common to the existing literature on human studies, given the limited availability of central nervous system cells. Moreover, as shown by Braun et al. that performed correlation of methylation between different tissues, a high concordance in blood and brain methylation signature was highlighted [23] supporting our choice of analysing epigenetics in blood cells. Finally, a major constraint of our study is the lack of replication in an independent cohort. Without validation in a separate sample, the robustness and reproducibility of our findings remain uncertain.

Our research presented some points of strength as well. Patients and healthy controls were matched for age and gender, providing a robust foundation for our analysis. This matching process mitigated potential confounding factors when assessing disparate methylation patterns.

Conclusions

This study represents the first investigation specifically focused on children with SNI, providing preliminary evidence for differential methylation patterns in pain-associated genes. Epigenetics may offer a promising avenue to overcome limitations in non-communicating children by providing objective biomarkers. Future perspectives should aim to further explore methylation patterns in relation to comorbid conditions and individual pain histories in order to deepen our understanding and improve clinical management.

Abbreviations

SNI	Severe Neurological Impairment
GMFCS	Gross Motor Function Classification System
IASP	International Association for the Study of Pain
ChAMP	Chip Analysis Methylation Pipeline
PCA	Principal component analysis
DMR	Differentially Methylated Regions
MSigDB	Molecular Signatures Database

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13052-026-02274-x.

Supplementary Material 1

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Author contributions

FC, LZ and FP designed the study. FC and SC performed the experiments. FS, GC, LDZ and FP enrolled the study's participants. FC and PDA analysed the data. The first draft of the manuscript was written by FC, LZ, AS and EB and all

authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Data availability
Data are available upon reasonable request. Our Excel database is available under request to the authors (***fulvio.celsi@burlo.trieste.it***).

Declarations

Ethics approval and consent to participate
The Independent Committee for Bioethics approved the research, following the Declaration of the World Medical Association (registration number IRB-BURLO 01/2022 09.02.2022). Informed consent was previously obtained from parents after full explanation regarding the study.

Consent for publication
All participants have agreed to have their data published in an aggregated, anonymized format.

Competing interests
Author AS serves as an Associate Editor for Italian Journal of Pediatrics. To ensure the integrity of the review process, the editorial workflow for this submission was handled independently by another editor, and the author had no involvement in the peer review or decision-making process related to this manuscript.

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