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RESEARCH

Whole Blood DNA Methylation Analysis Reveals Epigenetic Changes Associated with ARSACS

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Abstract
Autosomal recessive spastic ataxia of Charlevoix-Saguenay (ARSACS) is a rare inherited condition described worldwide and characterized by a wide spectrum of heterogeneity in terms of genotype and phenotype. How sacsin loss leads to neurodegeneration is still unclear, and current knowledge indicates that sacsin is involved in multiple functional mechanisms. We hence hypothesized the existence of epigenetic factors, in particular alterations in methylation patterns, that could contribute to ARSACS pathogenesis and explain the pleiotropic effects of *SACS* further than pathogenic mutations. To investigate this issue, we recruited eight patients affected by ARSACS, four characterized by early onset of the disease and four with late onset. We performed Whole Genome Bisulfite Sequencing using DNA from peripheral blood to define the methylome of patients and compared them with a control group. Our analysis showed that patients with ARSACS exhibit an altered methylation pattern and that the observed differences exist also among affected individuals with different age of onset. Our study provides valuable insights for employing epigenetic biomarkers to assess the severity and progression of this disorder and propels further investigations into the role of epigenetic processes in ARSACS pathogenesis.

Keywords Autosomal recessive spastic ataxia of Charlevoix-Saguenay · SACS · Sacsin · Whole genome bisulfite sequencing · WGBS · Methylome

Introduction

Autosomal recessive spastic ataxia of Charlevoix-Saguenay (ARSACS, OMIM #270550) is a frequent form of autosomal recessive ataxia described worldwide [1]. To date, more than 300 different pathogenic variants have been identified, associated with a well-known diversified spectrum of clinical features. Multiomic studies have shown that sacsin is a main actor in multiple molecular-level activities and biological pathways [2].

When combined, multiple and variegate clinical and functional data propose ARSACS as a very complex disorder with a high level of heterogeneity. This led us to hypothesize the presence of factors, such as epigenetics, that somehow contribute to disease etiology concomitantly and beyond the occurrence of mutations in the *SACS* gene. Currently, epigenetics is emerging as a crucial factor in understanding neurodegenerative processes, given the importance of epigenetic mechanisms for the development and functioning of the human brain [3, 4]. DNA methylation is one of the most studied epigenetic modifications, by which a methyl group is added to the 5th carbon of DNA cytosines by DNA methyltransferases. The conversion of cytosine to 5-methylcytosine typically occurs in cytosine-guanine dinucleotide sequences (CpG sites) and is involved in several cell processes including gene expression regulation, chromatin remodeling, and genomic stability [5, 6]. Alterations in DNA methylation have been associated with several neurological disorders, including inherited ataxias

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like Friedreich ataxia [7–9] ataxia-telangiectasia [10], and spinocerebellar ataxias [11–13]. However, DNA methylation has never been explored in ARSACS.

Recent studies highlighted that changes in DNA methylome can be captured directly in the whole blood of patients affected with neurological disorders, where they can also be correlated in some instances with clinical features, disease risk, and progression [14–16]. Thus, exploring the landscape of blood DNA methylation is attractive to clarify the molecular mechanisms underpinning neurological disorders and to gain valuable insights into clinical heterogeneity, potentially leading to improved diagnostic and therapeutic strategies.

This study aims to investigate the potential epigenetic role in ARSACS pathogenesis through Whole Genome Bisulfite Sequencing (WGBS) analysis, revealing the DNA methylation status across the entire genome, and to understand if it can contribute to explain the different phenotype severity existing among ARSACS patients.

Methods

Subjects

This study was approved by the Tuscany Regional Pediatric Ethics Committee. Eight patients with a clinical and genetic diagnosis of ARSACS (Table S1) were retrospectively recruited among the cohort of spastic-ataxic patients attending the research hospital IRCCS Stella Maris (Pisa, Italy). Patients were recruited based on disease onset: four with onset < 6 years (ARSACS early onset, hereinafter indicated as EO), and four with onset > 35 years (ARSACS late onset, hereinafter indicated as LO). Four healthy unrelated individuals were recruited as controls.

Whole Genome Bisulfite Sequencing

Genomic DNA was obtained using the MagPurix Blood DNA Extraction Kit 200 designed for the MagPurix DNA Extract (Zinexts, Zhonghe, Taiwan). Libraries for WGBS were prepared including a bisulfite treatment, during which unmethylated cytosines are converted into uracils, while methylated cytosines stay unchanged, and PE150 sequencing was performed using Illumina platform. Library preparation and sequencing were provided as custom service (Novogene, Cambridge, UK).

Raw Data Analysis

FastQC v0.11.5 was used to check the quality of raw data, and *Trimmomatic v0.36* [17] software for FASTQ pre-processing. GRCh38 was employed as reference genome. *RepeatMasker* was used to predict repeats, and *cpgIslandExt* to get CGI track. *Bismark* software v0.16.3 [18] was utilized to perform alignments of bisulfite-treated reads to the reference genome, and *Bowtie2* [19] to transform the reference genome into the bisulfite-converted version. Sodium bisulfite non-conversion rate was calculated as the percentage of cytosine sequenced at cytosine reference positions in the lambda genome initially fragmented together with genomic DNA.

Methylation Level (ML) and Differential Methylation Region (DMRs) Determination

The methylation level of individual CpG sites was calculated using the following formula: ML = mC/(mC + umC) (ML: methylation level; mC and umC: the number of reads supporting the methylated and unmethylated cytosines, respectively). Calculated ML was corrected with the bisulfite non-conversion rate [20].

Differentially Methylated Regions (DMRs) were identified using the DSS software [21] with the following parameters: smoothing.span = 200, delta = 0, p.threshold = 1e-05, minlen = 50, minCG = 3, dis.merge = 100, pct.sig = 0.5. DMRs were defined as genomic regions ≥ 50 bps and with ≥ 3 CpGs, with at least 50% of CpG sites being differentially methylated (mean methylation difference > 0, Wald-test p-value < 1e-05) between groups. Methylation levels of neighboring CpG sites (200 bps distance) were smoothed to account for spatial co-methylation.

Based on the distribution of DMRs throughout the genome, we identified genes associated with DMRs (DMR-related genes) as genes whose promoter regions (2 kb upstream of the transcription start site) overlap with the DMRs.

Enrichment Analysis

Kyoto Encyclopedia of Genes and Genomes (KEGG: http://www.genome.jp/kegg/) was applied for enrichment analysis of DMR-related genes [22]. KOBAS software [23] was employed to test the statistical enrichment of DMR-related genes in KEGG pathways. KEGG terms with corrected P-value less than 0.05 were considered significantly enriched.

Methylation Level of DMR in Individual Samples

The methylation level of ARSACS-DMRs and P-DMRs in individual samples was calculated by averaging the methylation levels of individual CpG sites within each considered region. For this calculation, only CpG sites with a

coverage ≥ 5x in all samples were considered. Regions that did not include any CpG sites meeting this criterion were excluded. This approach ensured that the methylation levels of DMRs were estimated consistently across all samples (by using a fixed set of CpG sites) and reliably (by including only CpG sites with sufficient coverage). Group-level methylation of DMRs was determined by averaging the methylation levels across all subjects within the same group.

Statistical Analysis

All statistical analyses were performed in *R* (version 4.3) with an alpha level set at 0.05. Principal component analysis (PCA) was performed with the *prcomp* function (*stats* library). A linear regression model was fitted using the *lm* function from the *stats* package in *R*. Elements were considered as deviating from the linear model if their residuals fell outside the range of the mean absolute residuals ± 2 standard deviations. The *ggplot2* and *ggvenn* packages were used for data visualization.

Results

DNA Methylation Profiles of ARSACS Patients Exhibit Broad Differences Compared to Controls

To investigate epigenetic changes linked to ARSACS, we performed WGBS on genomic DNA obtained from peripheral blood samples. We obtained a minimum of 301,755,277 sequencing reads and an average of 342,851,991 reads across our samples, of which 97% were retained after quality control, and 84% were uniquely mapped against the reference genome. We achieved an average coverage per cytosine site of 9,4x, thus ensuring a robust methylation call across most CpG sites. The bisulfite conversion efficiency, estimated by analyzing the conversion rate of unmethylated cytosines in a spiked-in control sample, was above 99% for all the samples, indicating a high conversion rate with minimal non-conversion artifacts. Per sample summary statistics of mapping efficiency and coverage metrics are reported in Table S2.

At first, we searched for Differentially Methylated Regions (DMR) between control subjects and all ARSACS patients. We identified 6,638 DMRs (ARSACS DMR) in ARSACS patients, of which 6,247 were located on autosomes (Table S3). These DMRs exhibited moderate differences in methylation level (median absolute differences of 0.11, Fig. 1a), with predominant hypomethylation in ARSACS patients (69.16% DMRs hypomethylated in ARSACS, Fig. 1b). When examining the distribution of ARSACS DMRs across the genome, we found that most were located in intronic regions, followed by CpG islands (CGI), promoters, repeated regions, exons, and CGI shores (Fig. 1c). Hypomethylated DMRs were prevalent in most genomic contexts (Fig. 1c). None of the identified ARSACS DMRs overlap with the *SACS* gene, arguing against a direct epigenetic regulation of the gene.

Then, we focused our attention on the 2,393 DMR located in the promoter region (2 kb upstream of the transcription start site) of 2,512 genes (Table S4). We therefore performed gene set enrichment analysis to explore whether these genes were involved in specific biological pathways. Among the top 20 enriched pathways (FDR p-value < 0.05), we found several processes previously associated with ARSACS, such as mitophagy, autophagy, endocytosis, cell signaling, axon development, calcium signaling, regulation of actin cytoskeleton, cell adhesion, and protein degradation (Fig. 1d, Table S5). According to the findings above mentioned, the pathways identified mostly involved hypomethylated genes (Table S5). Indeed, most of the top enriched pathways remained significant when considering hypomethylated and hypermethylated genes separately (Figure S1).

Overall, our results indicate that DNA methylation profiles of ARSACS patients exhibit broad differences compared to controls. Most of these differences are directed toward loss of DNA methylation in ARSACS subjects, and affected genes involved in biological pathways previously associated with ARSACS.

DNA Methylation at ARSACS DMRs Separates Subjects by Disease Status and Age-of-Onset

We explored whether DNA methylation at ARSACS DMRs could distinguish ARSACS patients from control subjects. To this aim, we computed the methylation level of ARSACS DMRs in each subject. In this analysis, we focused on 3,448 autosomal DMRs with sufficient coverage in all samples (at least 1 CpG site with coverage ≥ 5 reads in all samples, Table S6). Upon Principal Component Analysis (PCA) we observed that the methylation status of ARSACS DMRs consistently separated all patients from control subjects (Fig. 2a). Furthermore, patients with similar ages of onset seem to aggregate on the second principal component, except for one of the patients with early-onset ataxia (EO4 in Fig. 2a).

To explore the methylation pattern of ARSACS DMRs in early-onset (EO) and late-onset (LO) patients, we calculated the average methylation level of each DMR in EO, LO, and controls, and determined the relative DNA methylation change (difference from control average) for both the EO and LO groups. We found that DNA methylation changes observed in the two groups of patients were highly correlated (Pearson R coefficient 0.84, Fig. 2b). Regression

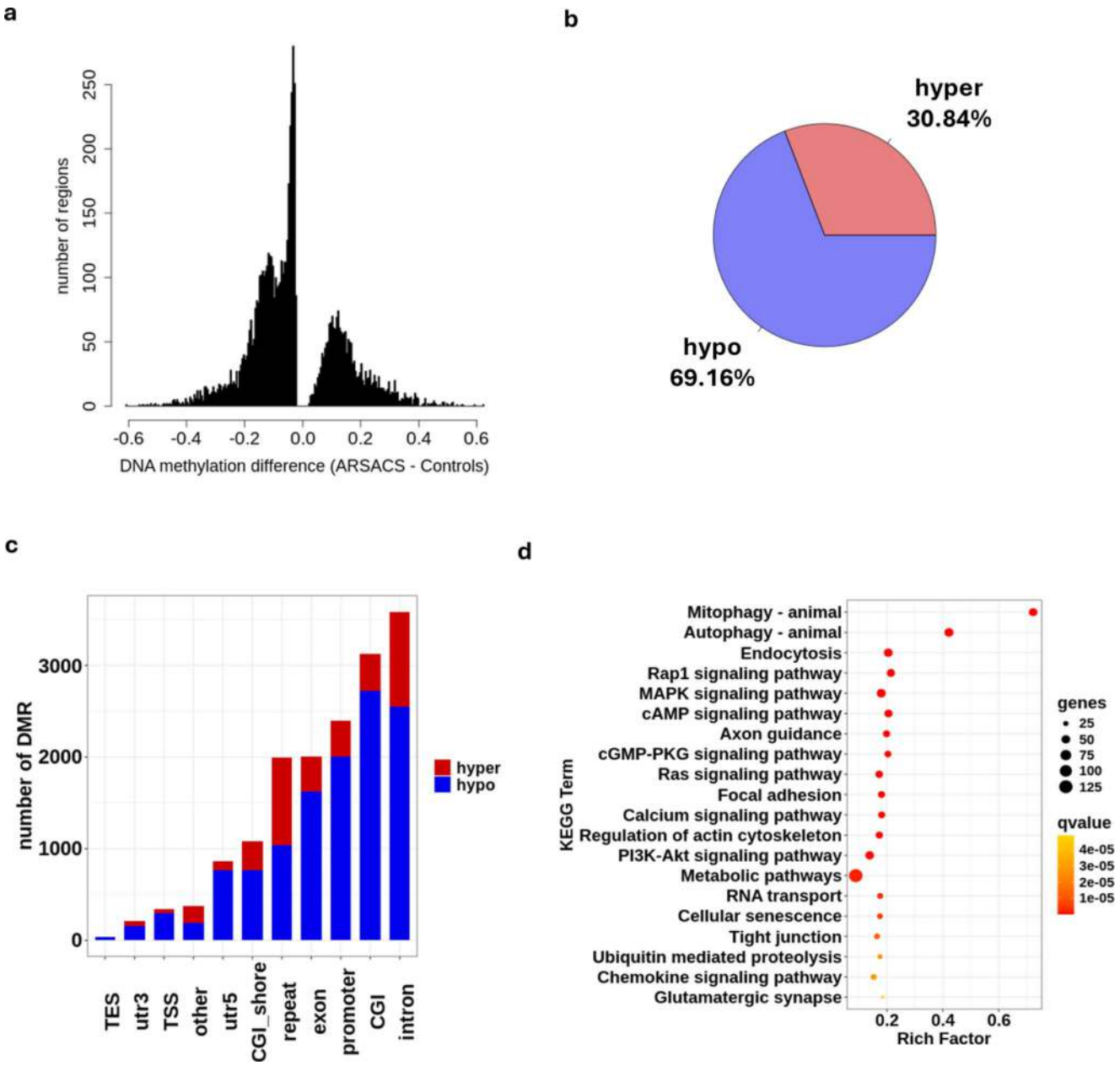

Fig. 1 **a** Mean methylation difference between ARSACS and controls at ARSACS DMRs. **b** Fraction of DMRs hypomethylated and hypermethylated in ARSACS patients. **c** Distribution of DMRs across genomic functional elements. TES, Transcription end site; TSS, Transcription start site. **d** Top20 KEGG Terms enriched for promoter DMRs

modeling revealed that most DMRs undergo almost equivalent changes in the two groups of patients (grey dots in Fig. 2b). Nonetheless, we observed 5% of DMRs ($n=189$) deviating from this general trend (red dots in Fig. 2b), meaning that these regions undergo more pronounced deviations from control DNA methylation in patients with either EO or LO ARSACS.

EO and LO Patients Exhibit Opposite Trend of DNA Methylation Changes Converging on Common Biological Pathways

We further investigated DNA methylation changes in EO and LO patients, and we found 5,530 and 5,072 DMRs in each group, respectively (Supplementary Table S7 and S8). The two sets of DMRs varied to similar extent from control DNA methylation (t-test p-value = 0.05, Fig. 3a). However, most LO DMRs (58.34%) were hypomethylated, thus

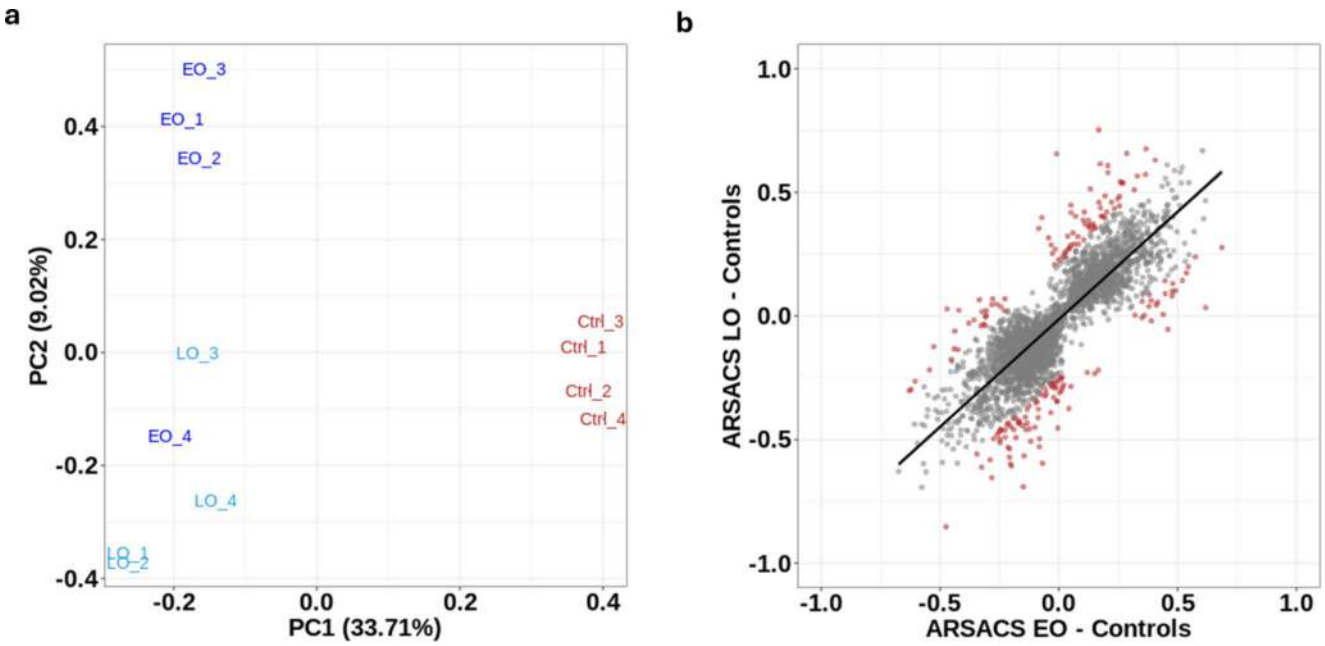

Fig. 2 **a** PCA plot of ARSACS and control subjects based on DNA methylation level of ARSACS DMRs (EO indicates subjects with early-onset ataxia, LO indicates subjects with late-onset ataxia, Ctrl indicates control subjects). **b** Plot of DNA methylation changes observed at ARSACS DMRs in the EO group (x-axis) and LO group (y-axis) compared to controls. (Regression line intercept −0.01, slope 0.87). Red dots indicate DMRs that significantly deviate from the linear model (residual outside the range of the mean absolute residuals ± 2 standard deviations)

following the trend described above in the ARSACS cohort, whereas the majority of EO DMRs (63.39%) were hypermethylated (Fig. 3b). None of the DMRs overlap with the *SACS* gene.

When focusing on promoter regions, we found 1,412 EO-DMRs located at promoters of 1,490 genes (Table S9) and 1,222 LO-DMRs located at promoters of 1,276 genes (Table S10). Gene set enrichment analysis highlighted that both EO and LO differentially methylated genes were involved in specific biological pathways (Fig. 3c and3d, Table S11 and S12). Interestingly, we found a large overlap among enriched processes in EO and LO groups, with 11 terms (Mitophagy, Autophagy, MAPK signaling, Calcium signaling, Endocytosis, cGMP-PKG signaling, Axon guidance, Rap1 signaling, cAMP signaling, RNA transport, Lysine degradation) among the top 20 enriched terms in both EO and LO. Furthermore, 10 of these terms (Mitophagy, Autophagy, MAPK signaling pathway, Calcium signaling, Endocytosis, cGMP-PKG signaling, Axon guidance, Rap1 signaling, cAMP signaling, RNA transport) were also among the previously described enriched terms in the overall ARSACS cohort (Fig. 1d).

These findings point to opposite trends in DNA methylation changes in patients with early or late-onset ARSACS, involving genes within shared biological processes.

Groups with Different Age of Onset Show Different Sets of Hyper- and Hypomethylated Genes

To connect the overlap observed on biological pathways with the discordant DNA methylation trend observed between EO and LO patients, we explored whether groups with different ages of onset exhibited diverse sets of differentially methylated genes in the main biological pathways identified. To pursue this goal, we selected genes with promoter DMRs contributing to the enrichment of KEGG terms in EO and LO.

Irrespective of disease onset, the number of hypomethylated genes was more than 7 times greater compared to hypermethylated genes (520 vs. 68, respectively; Fig. 4a), corroborating our previous finding about predominant hypomethylation in ARSACS patients (Fig. 1b). Furthermore, our analysis indicated that the EO group shows a higher number of hypermethylated genes compared to the LO group (166 vs. 72, respectively) and a lower number of hypomethylated genes (92 vs. 154, respectively). Moreover, our data indicated that most genes with promoter DMRs are unique to each group (Fig. 4b). These results indicate that discordant DNA methylation changes occurring in EO and LO ARSACS groups mostly involve different genes with opposite methylation changes.

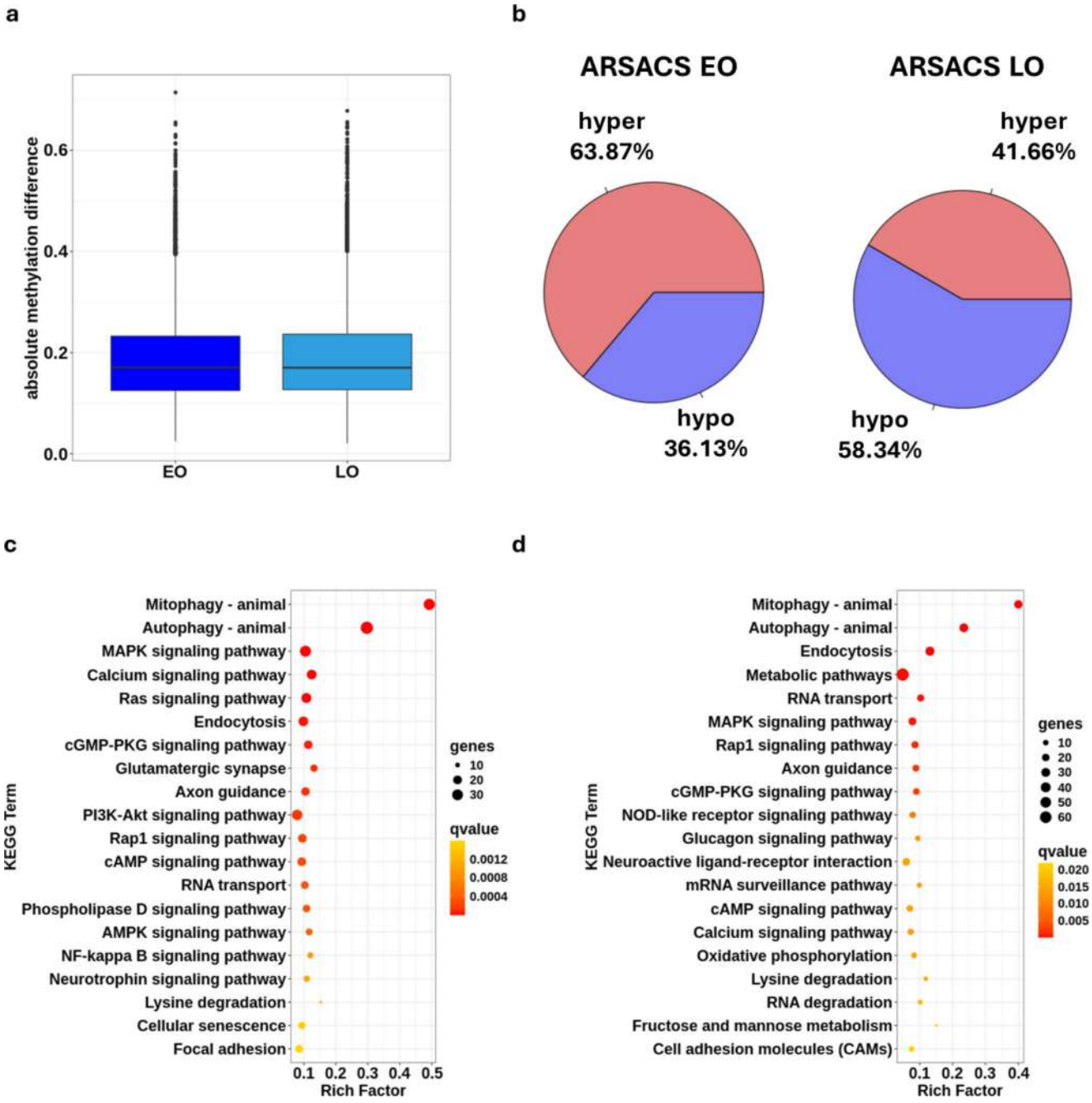

Fig. 3 **a** Absolute methylation difference observed for EO (left plot) and LO (right plot) DMRs. **b** Fraction of hypomethylated and hypermethylated DMRs in EO and LO patients. **c** Top20 KEGG Terms enriched for promoter DMRs in EO patients. **d** Top20 KEGG Terms enriched for promoter DMRs in LO patients

Early-Onset and Late-Onset Arsacs Patients Exhibit Both Common and Group-Specific DNA Methylation Changes at Gene Promoters

To comprehensively map the DNA methylation changes occurring at promoter regions in our ARSACS cohort, we analyzed the combined set of DMRs found in the overall ARSACS cohort, in EO, and in LO patients. We consolidated partially overlapping DMRs into larger, unique blocks, resulting in 3,688 distinct promoter regions with differential methylation in at least one of the comparisons (P-DMRs, Table S13). For each P-DMR, we annotated the specific group (ARSACS, EO, or LO) in which methylation changes occurred (Table S13, Fig. 5a). Focusing on P-DMRs with adequate coverage (1,674 P-DMRs containing at least one CpG site with 5x coverage across all samples), we quantified

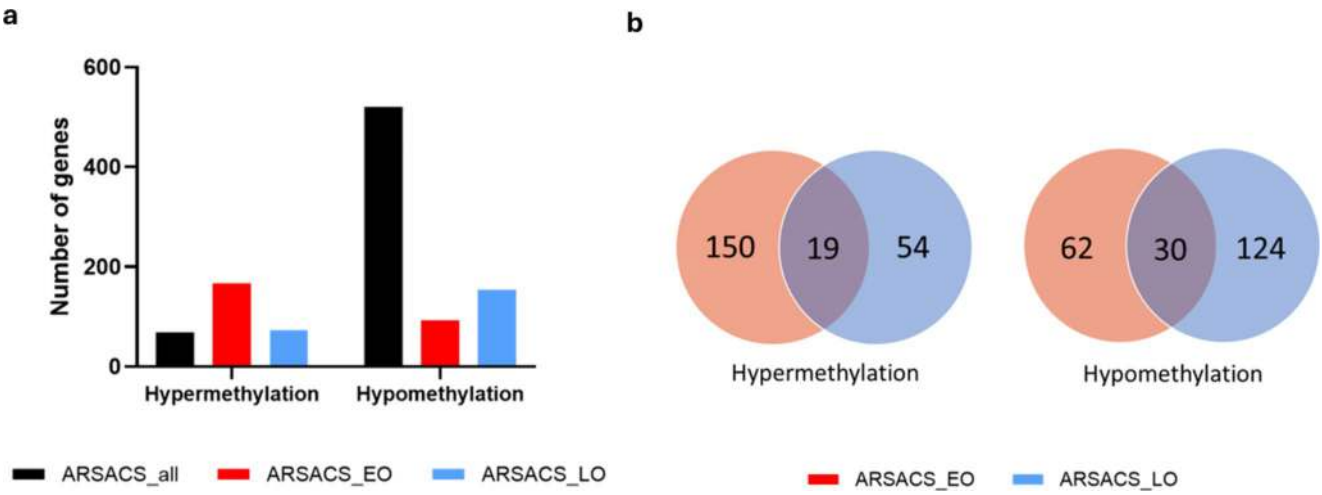

Fig. 4 **a** Number of genes with promoter DMRs in all ARSACS patients (ARSACS_all), in EO ARSACS patients (ARSACS_EO), and LO ARSACS patients (ARSACS_ LO) contributing to shared enriched KEGG terms. **b** Unique and shared genes contributing to shared enriched KEGG terms in EO and LO groups

the DNA methylation changes observed in EO and LO patients relative to control subjects (Table S14). Based on these characteristics, we described four groups of P-DMRs.

The first group included 1661 concordant regions (45.04% of P-DMRs). This group comprised 280 promoters with differential methylation in all comparisons (brown dots in Figure S2a) and 1,381 DMRs found only when comparing the entire ARSACS cohort to controls (gold dots in Figure S2a). Concordant regions changed to the same extent and in the same direction in EO and LO subjects, mostly showing a loss of DNA methylation (Fig. 5b). The pattern observed for these regions confirms that, despite different ages of onset, ARSACS subjects converge toward common epigenetic alterations.

The second group included 28 discordant regions, which accounted for less than 1% of P-DMRs. Discordant regions included DMRs shared between EO and LO groups but not found in the overall ARSACS cohort. These regions mostly showed DNA methylation changes in opposite directions in EO and LO subjects (Fig. 5c, Figure S2b).

The third set of regions included 1,091 EO-specific promoter regions, accounting for 29.58% of P-DMRs. This set encompassed the regions differentially methylated in EO but not in LO patients, including 288 regions overlapping with ARSACS DMRs. When investigating DNA methylation changes, we found most of these P-DMRs varied in the same direction in both EO and LO patients, being either hypo- or hyper-methylated in both groups (Fig. 5d, Figure S2c). However, the difference from control methylation was larger for the EO group (Fig. 5d). Notably, more than half of the regions underwent a gain of methylation in EO patients, according to the prevalent trend of hypermethylation previously described for this group (Fig. 5d).

The fourth set included 908 LO-specific promoter regions, accounting for 24.62% of P-DMRs. This set encompassed regions differentially methylated in LO but not in EO patients. As for the third group, these regions mostly changed in the same direction, varied to a larger extent in the LO group, and underwent a loss of methylation in LO patients (Fig. 5e, Figure S2d).

These findings suggest that ARSACS patients experience both common and group-specific DNA methylation changes. In terms of group-specific alterations, only a small portion of differentially methylated regions at gene promoters change in opposite directions. Instead, most regions undergo quantitative changes, meaning that they tend to be consistently hypo- or hyper- methylated, but to a larger extent in one group.

Enriched KEGG Terms Share a Similar Proportion of Common and Group-Specific DMRs

Finally, we investigated whether the previously identified groups of P-DMRs were evenly distributed among the enriched KEGG terms. We focused on processes commonly enriched in EO and LO groups and categorized the 377 DMR-related genes according to the group of their P-DMR (Table S15). We found that 45.08% of the genes had concordant P-DMRs, whereas 29.01% of genes had EO-specific P-DMRs, and 24.94% of genes had LO-specific P-DMRs. Only 4 genes (<<1%) harbor P-DMRs with discordant changes between EO and LO subjects (Fig. 6a). Notably, we did not find any difference between the proportion of the three major groups of genes across the 10 enriched terms (chi-square test p-value 0.79, Fig. 6b).

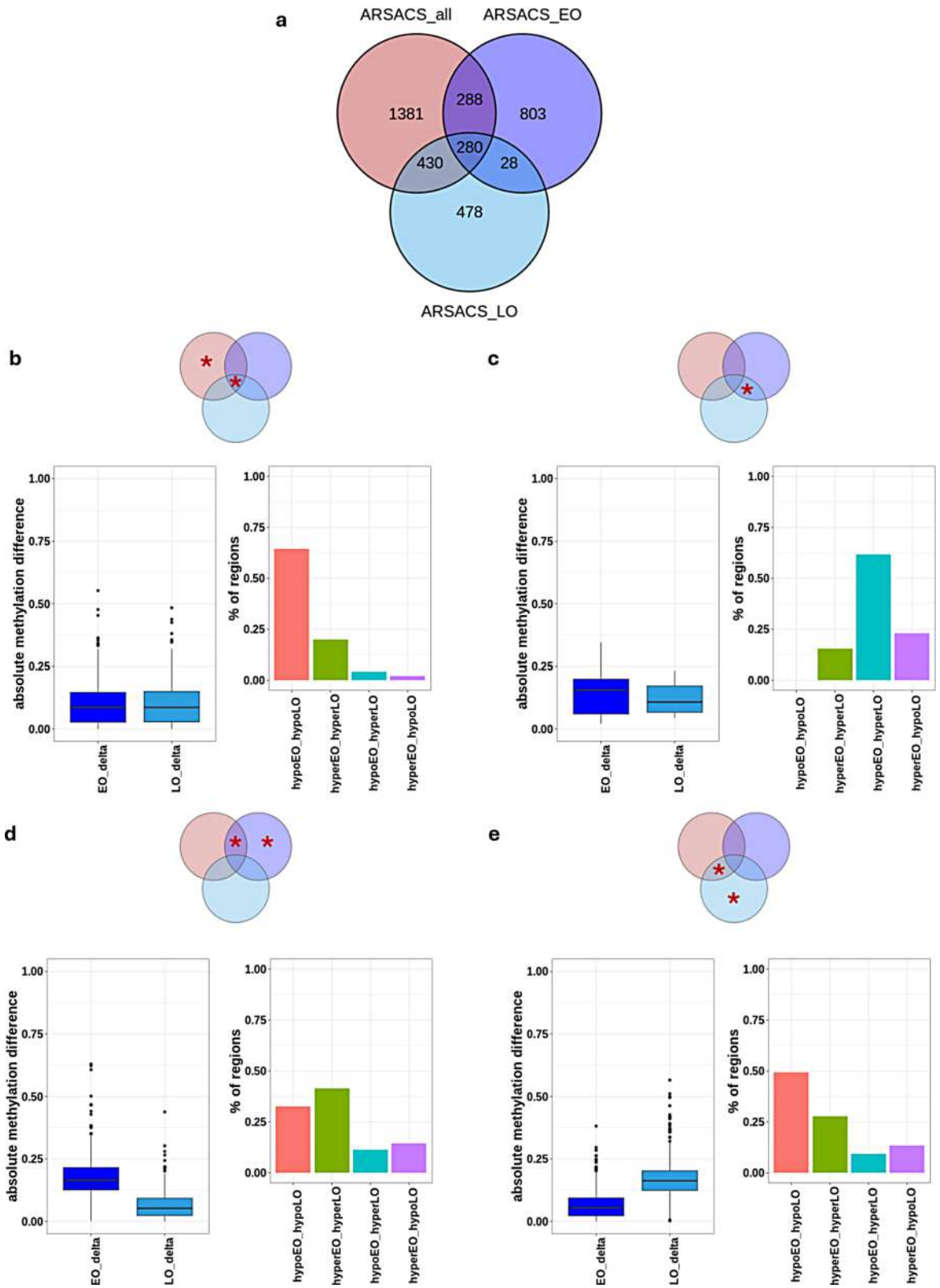

Fig. 5 **a** Overlap of P-DMRs identified in the overall ARSACS cohort, in EO and LO patients. **b-e**. boxplots showing absolute DNA methylation difference from controls in EO and LO patients (left plot), and barplots showing the fraction of regions with DNA methylation changes occurring in the same direction (hypoEO_hypoLO, hyperEO_hyperLO) or opposite direction (hypoEO_hyperLO, hyperEO_hypoLO) in EO and LO patients. **b** Concordant P-DMRs. **c** Discordant P-DMRs. **d** EO-specific P-DMRs. **e** LO-specific P-DMRs

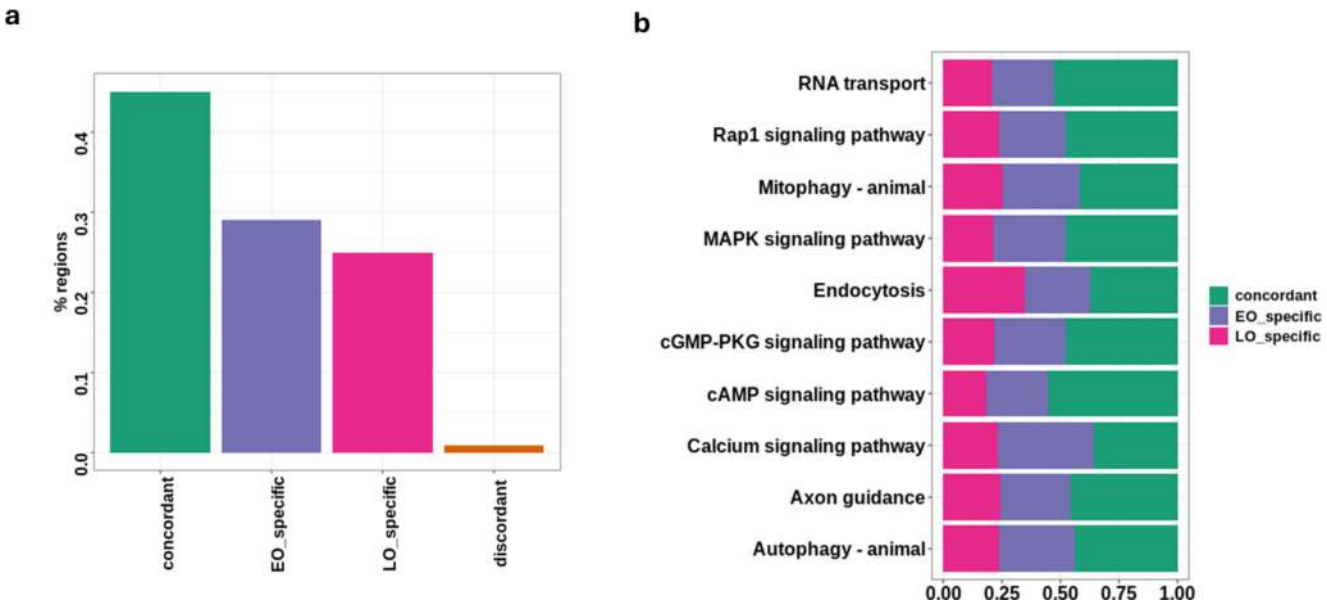

Fig. 6 **a** Fraction of genes with concordant, EO-specific, and LO-specific P-DMRs for KEGG terms enriched in both EO and LO groups. **b** Fraction of genes with concordant, EO-specific, and LO-specific P-DMRs for each KEGG term

These results suggest that epigenetic changes occurring equally in all ARSACS patients coexist with group-specific epigenetic changes in enriched biological pathways.

Discussion

In this study we analyzed genome-wide DNA methylation in a cohort of ARSACS patients, providing the first analysis of global changes in blood-DNA methylation profiles induced by *SACS* mutations that are causative of ARSACS.

We identified several regions undergoing significant DNA methylation changes compared to control subjects. The prevalent direction of DNA methylation changes was toward hypomethylation in ARSACS patients and resulted to be more evident in the LO group rather than the EO group. A similar finding was reported in a recent study involving late-onset Alzheimer's disease patients, in which most of the differentially methylated promoters were hypomethylated [24], an outcome further confirmed by another study [25].

In terms of genomic functional elements, most DMRs were located in introns, followed by CpG islands (CGI), promoters, repeated regions, exons, and CGI shores. We focused our analysis on CpG islands and promoters, which are known to be modulated by epigenetic mechanisms. Although the impact of perturbed DNA methylation at the CGI and CGI-flanking regions in ARSACS is yet to be understood, it is worth of note that hypomethylation in these regions in certain genes has been described in patients with Alzheimer's disease [26].

However, we cannot exclude a role for DMRs located in the other functional contexts. Indeed, evidence exists that gene-body DNA methylation can regulate gene expression levels by suppression of transcriptional noise [27, 28], choice of alternative initiation sites [29], and modulation of alternative splicing [30]. Methylation of repeat sequences is also a known mechanism for silencing transposable elements such as LINE1 and Alu elements [31], whose role in neurodegeneration has been described in Alzheimer's disease and other neurological disorders [32, 33]. In addition, a recent report describing LINE1 RNA as an important regulator of DNA methylation in promoters of genes involved in cortical development [34] could add yet another level of complexity to this scenario.

Notably, we found that DMRs were enriched at promoters of genes involved in specific biological pathways. Among the top significant pathways, the role of autophagy and mitophagy in ARSACS has been already widely described by our group and others [35–38]. Vesicle trafficking including endocytosis and the involvement of cell adhesion in ARSACS pathogenesis were also among the altered significant pathways and have been described in *SACS* knock-out cell and mouse models with significant consequences on axonal development [39]. Our recent proteomic study performed in ARSACS patients' fibroblasts further confirmed these findings [40]. Moreover, several signaling pathways were found to be altered in our analysis, including that related to calcium. Remarkably, calcium homeostasis has been implicated in the degeneration of Purkinje cells in *SACS* knock-out mice and confirmed by our group [40, 41]. Yet according to our findings achieved in ARSACS

fibroblasts [40], our methylation analysis showed regulation of actin cytoskeleton as significantly represented amidst altered processes.

Our study adds ARSACS to congenital ataxias for which altered DNA methylation can be described. Indeed, locus-specific epigenetic changes have been previously described in other hereditary ataxias, such as FRDA, SCA2, and SCA3, and directly coupled to disease-causing triplet expansions [7, 11, 12]. Genome-scale DNA methylation changes were instead described in autosomal dominant cerebellar ataxia with deafness and narcolepsy [42, 43], Ataxia-telangiectasia [10], and in SCA3 [13]. Notably, although DNMT1 is a characterized component of the epigenetic machinery [44], and involvement in chromatin remodeling has been described for ATM [45] and ATXN3 [46], a role of sacsin in chromatin dynamics has never been reported. However, considering the pleiotropic roles of sacsin, we could speculate that DNA methylation changes here described reflect the impairment of downstream processes, and possibly the activation of an adaptive response to sacsin deficiency.

Upon principal component analysis, we found that epigenetic profiles consistently discriminated patients from control subjects, and also separated patients based on disease onset, although an EO patient (ARSACS_EO_4) clustered together with LO. This could be due to a slower disease progression, or to a later sampling age compared to other EO patients. Considering the latter hypothesis, it could hence be possible that the methylation profile could be similar to that of the late-onset patients yet in early adulthood.

Further investigation of DNA methylation profiles considering disease onset enabled us to pinpoint genomic regions undergoing either consistent changes in all ARSACS patients or differential epigenetic rewiring in patients with diverse disease onset. In this regard, DNA methylation was previously associated with clinical heterogeneity in FRDA patients, where the degree of hypermethylation in intron 1 of the *FXN* gene was found to be directly correlated with the length of triplet expansion and inversely correlated with residual *FXN* expression and disease onset [8, 9]. Similarly, the degree of methylation in the *ATXN2* promoter was correlated with the disease onset in SCA2 homozygotes and SCA3 patients [11]. Interestingly, the same study described different levels of methylation associated with disease anticipation in a SCA2 pedigree with stable CAG repeat length. At the genome scale, an acceleration of epigenetic age was described in SCA3 and correlated with disease onset [47]. Distinctive gene expression and DNA methylation patterns were also reported in patients with mild or classic forms of ataxia-telangiectasia [10]. By highlighting epigenetic changes possibly associated with age of onset of ARSACS, our study highlights interesting perspectives for exploring epigenetic biomarkers of disease severity and progression for this disorder.

Remarkably, we identified a core group of biological pathways in which concordant changes occurring in all ARSACS patients coexist with group-specific epigenetic changes. Although we cannot rule about the functional impact of epigenetic changes on these biological processes, we can hypothesize that group-specific epigenetic modifications may reflect the extent to which molecular mechanisms are compromised or activated as adaptive responses upon sacsin deficiency. Furthermore, despite our analysis shows that limited differences exist among patients with different age of onset in terms of biological pathways involved, our data also indicate that most genes with DMRs are unique to each group.

We also observed that EO and LO patients apparently show distinct trends in global DNA methylation, with prevalent hypermethylation in EO. However, when examining DNA methylation in the reciprocal subgroup, we found that discordant DNA methylation changes account for a minor fraction (less than 1%) of all DMRs identified in this study. Instead, we observed that DNA methylation tends to shift in the same direction in EO and LO patients.

This indicates that hypermethylation is more pronounced in EO patients rather than representing a fully divergent trend. Further studies are needed to determine whether these changes represent an onset-associated epigenetic background, potentially acting as phenotype modifiers, or if they reflect a distinct disease progression route in EO patients.

While our study primarily focuses on identifying differential methylation in ARSACS, we propose that these changes could influence gene expression in pathways critical to neuronal health, such the mitochondrial function [35, 36] and intracellular calcium signaling, as recently described [40, 41]. In this context, epigenetic alterations might act as modifiers that, in combination with *SACS* mutations, modulate disease severity and progression. Alternatively, these methylation changes may accumulate as result of disrupted molecular homeostasis associated with ARSACS, representing an intermediate epi-phenotype of the disease. Lastly, they could reflect adaptive responses, where cells attempt to compensate for SACS deficiencies through regulatory mechanisms. Confirming these hypotheses will require further functional and multi-omic studies. Furthermore, we investigated the sacsin interaction network using STRING [48], and our analysis did not reveal any DMRs in the promoter regions of the main interactors within the sacsin functional network (namely ATXN1, DNAJB2, DNM1L, HSPA1A, HSPA1B, HSPA4, NWD2, PICK1, SGCG, and UBE3A). This suggests that direct epigenetic regulation may not be a primary mechanism within the sacsin interaction network in ARSACS.

In this study, we used whole-genome bisulfite sequencing (WGBS) to comprehensively characterize DNA methylation across the entire genome. Although WGBS inherently limits the sample size, we prioritized capturing the broadest possible range of epigenetic changes in a pilot study to provide initial evidence of epigenetic dysregulation in ARSACS. This approach comes at the cost of limited coverage of inter-individual heterogeneity among patients and poses challenges in interpreting outlier characteristics. For instance, one early-onset subject exhibited a DNA methylation pattern most similar to late-onset subjects. Expanding the study to a larger cohort will better discriminate samples fitting within the spectrum of epigenetic changes in ARSACS from those with outlier characteristics. However, ARSACS is predominantly an early-onset disease, thus rendering very challenging the recruitment of patients with late-onset since they are extremely rare [2]. Nonetheless, despite the limited sample size, our study has the advantage of exploring the DNA methylation encompassing both early- and late-onset patients.

Another limitation of this study is that the healthy individuals in the control group were only partially matched with the ARSACS subjects in our cohort. More specifically, the age range of the control subjects aligned more closely with late-onset ARSACS patients rather than the early-onset group, mainly, with obvious challenges to obtain samples and ensure compliance from age-matched healthy donors at pediatric age. Additionally, there is a partial sex match between the control group (2 females and 2 males) and late-onset ARSACS patients (3 females and 1 male). However, the mostly concordant changes between early and late-onset ataxia patients, along with their convergence toward common epigenetic changes and disrupted pathways, suggest a limited confounding effect from these covariates.

In summary, we described epigenetic changes occurring in the blood of ARSACS patients. Although it has been shown that blood and brain DNA methylomes overlap to a certain extent [49, 50], further studies will be needed to clarify the extent to which epigenetic alterations identified in this study fully capture epigenetic changes established in sacsin-deficient neurons. Nonetheless, it is worth pointing out that an increasing corpus of studies highlights DNA methylation as a valuable source of biomarkers for brain phenotypes [51–54] and brain-related disorders [24, 55–57]. Therefore, blood is generally recognized as a suitable surrogate tissue to study the epigenetics of brain disorders.

Finally, we believe this study opens valuable new avenues for ARSACS research. Firstly, it indicates that DNA methylation analysis could support ARSACS diagnosis. Larger studies including both ARSACS patients and patients affected with other ataxias will assess the feasibility of an ARSACS-specific episignature—a set of CpGs whose methylation status is both sensitive and specific to ARSACS. Secondly, DNA methylation may provide biomarkers for disease severity and progression, potentially extending to monitor treatment response. Finally, evaluating the functional impact of DNA methylation on biological pathways could reveal new pharmacological targets to modulate disease progression. Notably, some pathways implicated in our findings, such as mitophagy and calcium signaling, are indeed druggable, with modulation showing molecular-level effects in ARSACS models [37, 41].

Conclusion

We analyzed whole-genome DNA methylation in blood samples obtained from a cohort of ARSACS patients and healthy controls. We identified multiple regions experiencing notable DNA methylation alterations compared to control subjects, supporting the theory that epigenetics could play a role in ARSACS. We also described peculiar epigenetic changes of patients with early and late-onset ARSACS, and thus putatively associated with age of onset. Of note, the identified epigenetic changes converge on pathways known to be perturbed upon sacsin deficiency. Further studies on a larger cohort will enable the validation of the findings of this study.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s12311-025-01791-5.

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Author Contributions GDR: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Software, Validation, Visualization, Writing – original draft. VN: Visualization, Writing – review & editing. DD: Visualization, Writing – review & editing. SD: Visualization, Writing – review & editing. FMS: Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing. DG: Conceptualization, Funding acquisition, Investigation, Project administration, Resources, Visualization, Writing – original draft, Writing – review & editing.

Data Availability The data supporting the findings of this study are available as Supplementary Materials. Raw data are available from the corresponding authors upon reasonable request.

Declarations

Ethics Approval The study was approved by the Tuscany Region Ethics Committee. The patient gave informed consent for diagnostic

testing and research studies. The study was conducted in accordance with the Helsinki Declaration of 1964, as revised in October 2013 in Fortaleza, Brazil.

Competing Interests The authors declare no competing interests.

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