UNIVERSITÀ DEGLI STUDI DI MILANO

DOCTORAL PROGRAMME IN NUTRITIONAL SCIENCE

IMPACT OF KETOGENIC DIET TREATMENT IN GLUT1 DEFICIENCY SYNDROME: DISCOVERY OF MOLECULAR MECHANISMS RESPONSIBLE FOR PHENOTYPE CHANGES IN BOTH PEDIATRIC AND ADULT PATIENTS

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4.5.4.1 Comparison between lncRNA-correlated genes and DEGs

The top 25 genes positively and negatively correlated with the identified lncRNA have been compared with 69 DEGs detected in adults versus children study. This highlighted some deregulated RNAs associated with three lncRNA, including PAX8-AS1, ENSG00000260772 and RMRP.

The upregulated TG gene, encoding a glycoprotein homodimer produced predominantly by the thyroid gland, is implicated in positive correlation with upregulated lncRNA PAX8-AS1. Studies on PAX8-AS1 are limited and primarily focus on its potential regulatory role in gene expression and its potential implications in cancer biology and in in various pathological conditions (Zhou et al., 2021).

Otherwise, the ribonucleoprotein RN7SL2 is downregulated in adults and predicted to be associated in negative correlation with upregulated lncRNA ENSG00000260772, involved in mRNA surveillance pathway.

Moreover, the downregulated lncRNA RMRP showed a positive correlation with 10 downregulated ncRNA, involving the RPPH1, RN7SK, RN7SL2, SCARNA12, SNORD3A, SCARNA10, SNORA73A, SCARNA13, ENSG00000259001 and SNORA48 DEGs. Interestingly, RMRP is defined as T cell-related lncRNA in epilepsy (Sharifi et al., 2022), contributing to the pathogenesis of epilepsy through immune-related and -unrelated mechanisms, as reported by Sharifi *et al*. (2022).

4.5.5 Identification of isoforms switching events

To further characterize the dysregulation observed in RNA splicing processes and given its documented impact on neuro-excitability in the literature (Iijima & Yoshimura, 2019; Lopez Soto et al., 2019), we analyzed variations in splicing events and isoform levels.

The differential isoform expression analysis in adults versus children with GLUT1-DS highlighted 70 differentially expressed isoforms with switching features associated to 62 unique genes, which were mostly protein coding genes (*Table 12*). Given the abundance of isoforms identified with the above analysis, we first investigated the molecular mechanisms underlying these changes through the consequence enrichment splicing analysis and for each type of splicing, the total number of events detected. The switched isoforms are involved in multiple alternative splicing (AS) events, observing that the AS events are not equally used. As reported in *Figure 27a*, when considering the number of AS events, Alternative Transcription Start Sites (ATSS) and Termination Sites (ATTS) are the most predominant with 131 and 135 events respectively, followed by exon skipping (83 ES events), alternative 5′ donor sites (77 A5 events), alternative 3′ acceptor sites (70 A3 events), multiple exon skipping (39 MES events), intron retention (29 IR events) and mutually exclusive exons

(MEE) with only 1 event. When considering opposite consequence analysis (e.g. the gain versus loss of ATSS), shown in *Figure 27b*, the majority of changes by looking for the estimates most different from 0.5 are in regard to the use of IR and A5.

Once we analyzed the mechanisms underlying the changes observed, we focused our attention on the different isoform switches highlighted by the differential analysis. As reported by *Figure 27c*, the most frequent changes are the ones affecting protein domains (49 events), coding potential (36 events), intrinsically disordered regions (25 IDR events) and intron retention (25 events). Indeed, as result of the transcriptional deregulation, the genes undergo mostly a loss in intron retention, gain in protein domains and IDR and above all transcripts are mostly coding, confirming the results highlighted by the differential expression analysis. *Figure 24d* highlighted an uneven distribution of impacts, e.g. more loss than gain in intron retention, which we deeply and systematically investigated for all consequences using the built-in enrichment analysis. For each pair of opposite consequences (e.g., protein domain loss vs gain and so on) reported on the y-axis, the analysis highlights the fraction of switches, affected by either of the opposing consequence, that results in the consequence indicated (e.g. protein domain loss) reported in x-axis. If the fraction is significantly different from 0.5, there is a systematic bias in isoform switch consequences. According to this analysis, many of the opposing consequences (domain loss and intron retention loss) are significantly unevenly distributed, suggesting that these consequences are specific for the adult with GLUT1-DS.

KRI1	TMEM161A	ZNF691	ENSG00000247121
FAM156B	CRADD	RCCD1	RWDD4
DPP3-DT	COG6	ZNF92	POLR2I
FAM3A	CBLL1-AS1	CBY1	LTF
MRPS7	PIGS	GALK1	COMTD1
ECT2	FAM185A	RAP2C	EIF2AK2
STK19	TMCO4	FKBP5	MCAT
IMMT	HLA-DQA1	KIF9-AS1	SMIM13
EXOSC7	MRM3	NACC1	TAF4
ZNF419	MRPL19	HCFC1	COPG2
SMAGP	OPA3	C12orf43	MTX2
CD3E	IDNK	POLR2J3	HPF1
METAP2	HDHD2	RNF214	EGLN3
TRMT1L	SLC9A6	GEMIN2	SUOX
ARL17B	ZNF573	ZNF496	
VSTM1	LAS1L	APOO	

Table 10: Switching genes involved in differential isoform expression in adults versus children with GLUT1-DS.

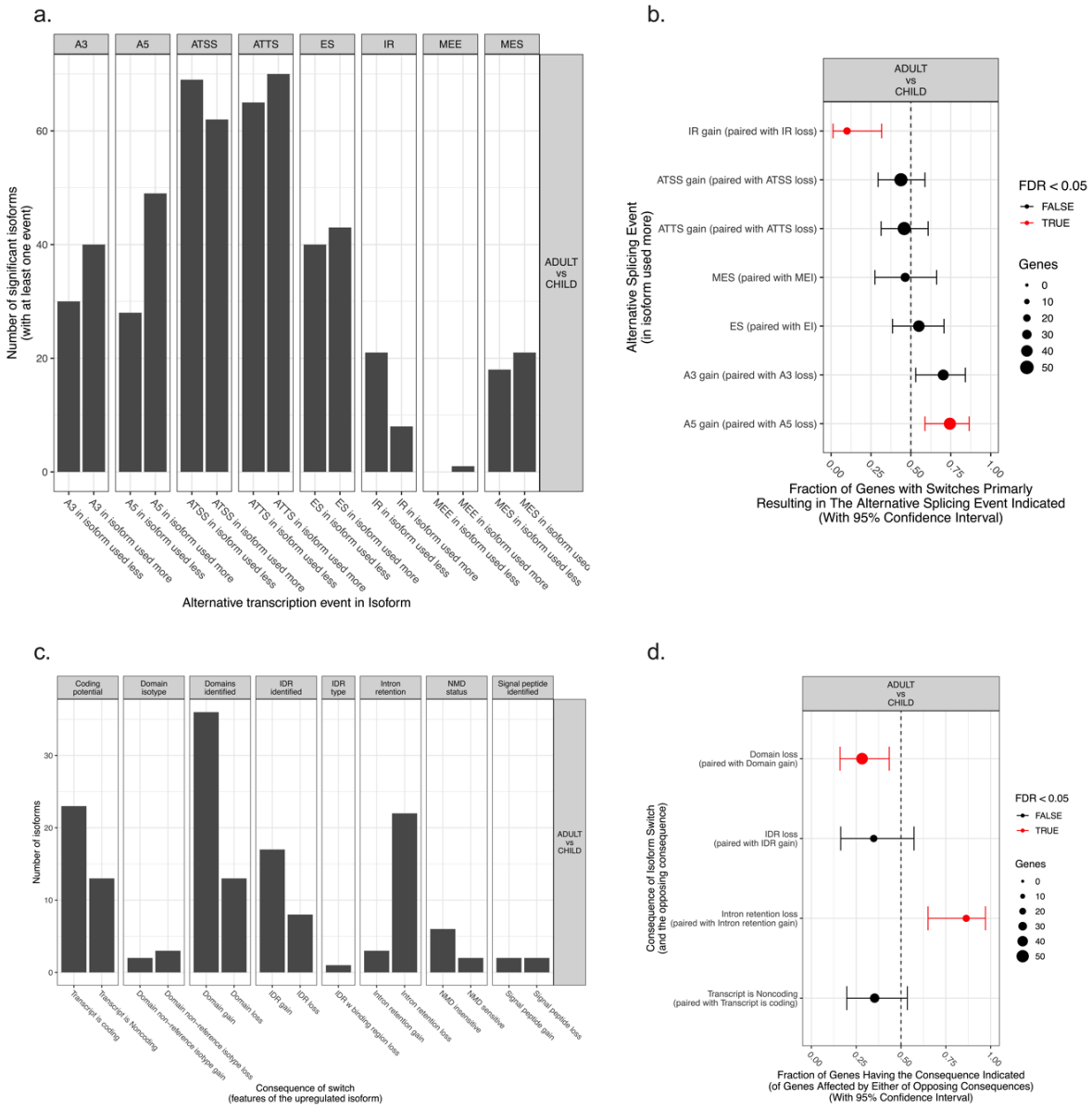
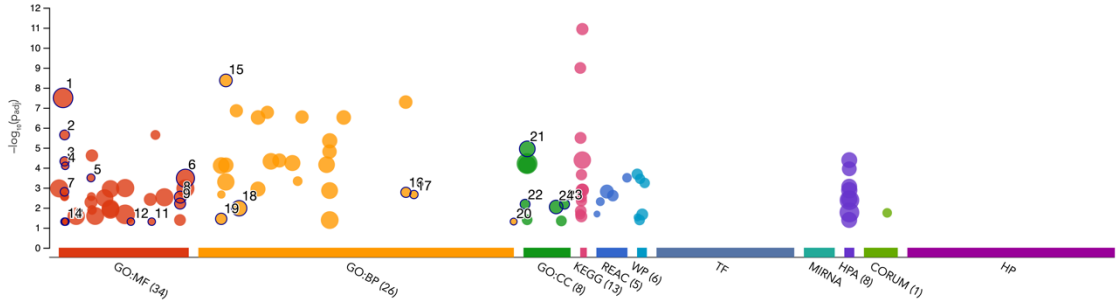


Figure 27: Transcripts analysis for switched isoforms between adults and children; a, b) Distribution of isoform usage in each type of alternative splicing; c, d) Overview of the number of switched isoforms predicted to have functional consequences.

4.5.5.1 Biological implications of switching genes

Given the evidence in alternative splicing and switching isoform analysis, we performed a functional enrichment analysis on all switching genes involved in differential isoform expression to further characterize molecular mechanisms at the basis of the dysregulation. The analysis performed on g:Profiler webtool returned a total of 24 pathways shown in *Figure 28*. Interestingly, these pathways include metabolic pathways such as fatty acid oxidation, acetyl-CoA process and mitochondrial pathways.



ID	Source	Term ID	Term Name	p_{adj} (query_...
1	GO:MF	GO:0003824	catalytic activity	3.207×10^{-8}
2	GO:MF	GO:0003995	acyl-CoA dehydrogenase activity	2.289×10^{-6}
3	GO:MF	GO:0003993	acid phosphatase activity	4.776×10^{-5}
4	GO:MF	GO:0004060	arylamine N-acetyltransferase activity	8.049×10^{-5}
5	GO:MF	GO:0016401	palmitoyl-CoA oxidase activity	3.208×10^{-4}
6	GO:MF	GO:1901363	heterocyclic compound binding	3.352×10^{-4}
7	GO:MF	GO:0003985	acetyl-CoA C-acetyltransferase activity	1.592×10^{-3}
8	GO:MF	GO:0140359	ABC-type transporter activity	2.945×10^{-3}
9	GO:MF	GO:0140326	ATPase-coupled intramembrane lipid transport...	6.212×10^{-3}
10	GO:MF	GO:0003994	aconitate hydratase activity	4.964×10^{-2}
11	GO:MF	GO:0051538	3 iron, 4 sulfur cluster binding	4.964×10^{-2}
12	GO:MF	GO:0046623	sphingolipid floppase activity	4.964×10^{-2}
13	GO:MF	GO:0004085	butyryl-CoA dehydrogenase activity	4.964×10^{-2}
14	GO:MF	GO:0003989	acetyl-CoA carboxylase activity	4.964×10^{-2}
15	GO:BP	GO:0006635	fatty acid beta-oxidation	4.306×10^{-9}
16	GO:BP	GO:0072350	tricarboxylic acid metabolic process	1.694×10^{-3}
17	GO:BP	GO:0090131	mesenchyme migration	2.180×10^{-3}
18	GO:BP	GO:0009410	response to xenobiotic stimulus	1.060×10^{-2}
19	GO:BP	GO:0006084	acetyl-CoA metabolic process	3.588×10^{-2}
20	GO:BP	GO:2001295	malonyl-CoA biosynthetic process	4.910×10^{-2}
21	GO:CC	GO:0005759	mitochondrial matrix	1.136×10^{-5}
22	GO:CC	GO:0001401	SAM complex	6.655×10^{-3}
23	GO:CC	GO:0140275	MIB complex	6.655×10^{-3}
24	GO:CC	GO:0072562	blood microparticle	9.147×10^{-3}

version	e111_eg58_p18_f463989d
date	29/11/2024, 21:53:27
organism	hsapiens

g:Profiler

Figure 28: Enrichment analysis for GO on switching genes. The upper graph represents the number of terms involved for each biological analysis, the table shows ID and name of the pathways.

4.5.5.2 Significant alternative splicing isoforms

A bibliographic analysis of previous literature was performed, to identify how many genes, amongst the 62 found, had been associated with GLUT1-DS and/or epilepsy. This analysis revealed that only three switching genes, including *SUOX*, *NACC* and *HLA-DQA1*, had been previously linked with the epilepsy condition.

SUOX gene encodes the sulfite oxidase, a homodimeric enzyme localized to the intermembrane space of mitochondria for oxidation of sulfite to sulfate, the final reaction in the oxidative degradation of the sulfur amino acids cysteine and methionine. Besides the involvement in metabolism of amino acids and derivates, WikiPathways analysis highlighted pathways such as metabolic epileptic disorders (WP5355) and ethylmalonic encephalopathy (WP5030) (Sass et al., 2010). The isoform switch analysis for this gene illustrated in *Figure 29* showed no difference in *SUOX* expression between adults and children's samples, but a significant switch in isoform usage across conditions is marked. By comparing which isoforms are changing to the isoform structure, it can be inferred that

in the adults' samples the short intracellular non-canonical transcript (ENST00000394109.7) is used.

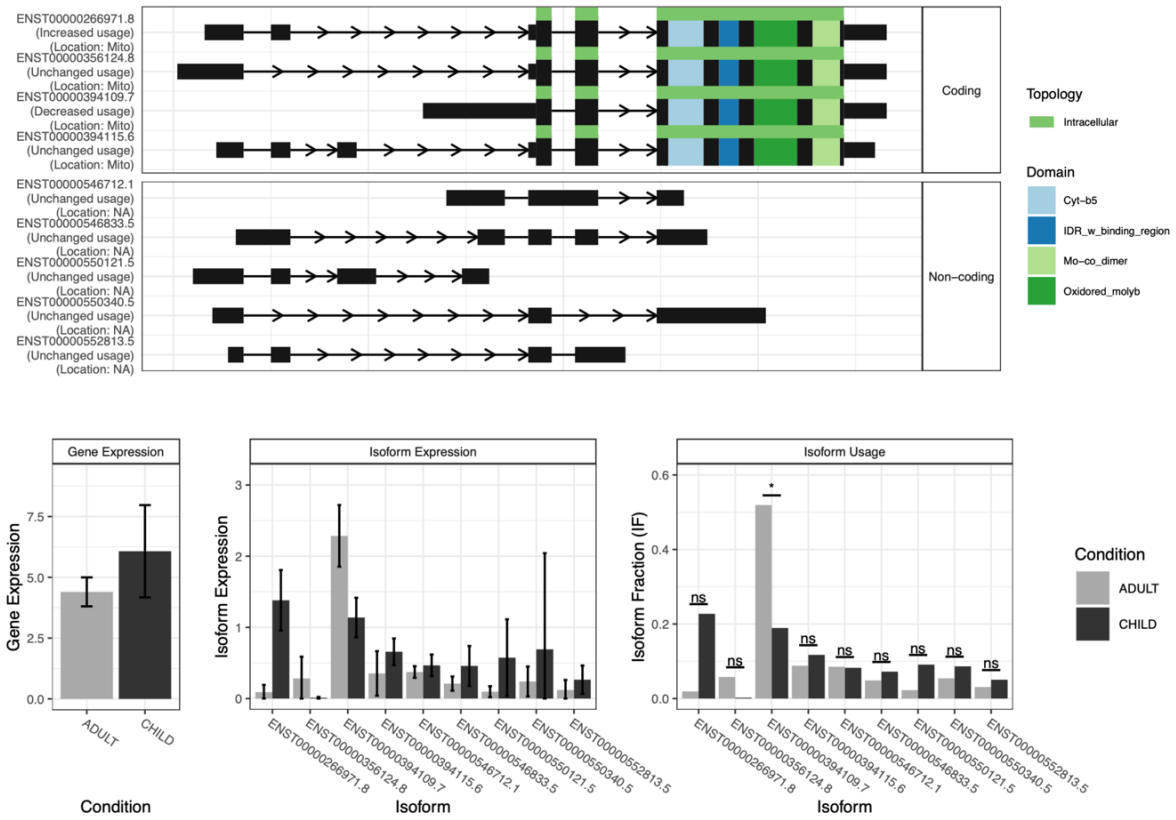


Figure 29: The isoform switch in SUOX gene using the switchPlot functionality.

NACC1 gene, encoding a member of the BTB/POZ protein family, acts as a transcriptional repressor in neuronal cells through recruitment of HDAC3 and HDAC4. The disease associated in OMIM include a neurodevelopmental disorder characterized by seizures, microcephaly and global developmental delay (OMIM #617393) (Schoch et al., 2017). In terms of isoform analysis, no difference in the gene expression of *NACC1* is highlighted in the graph in *Figure 30*. A significant switch in isoform usage across conditions is found. Indeed, the protein-coding transcript ENST00000586171.3 is used more in pediatric samples.

The *HLA-DQA1* gene belongs to the HLA class II alpha chain paralogues, and it plays a central role in the immune system by presenting peptides derived from extracellular proteins. Reactome analysis revealed the Interferon Signaling (R-HSA-913531), Adaptive Immune System (R-HSA-1280218) and Cytokine Signaling (R-HSA-1280218) as biological pathways for *HLA-DQA1* gene (Horta et al., 2015). From *Figure 31*, no difference in the gene expression is noted, but a significant switch in isoform usage between adults and children's samples is found. Specifically, the protein-coding isoform ENST00000374949.2 is more used in adults when compared with pediatric patients.

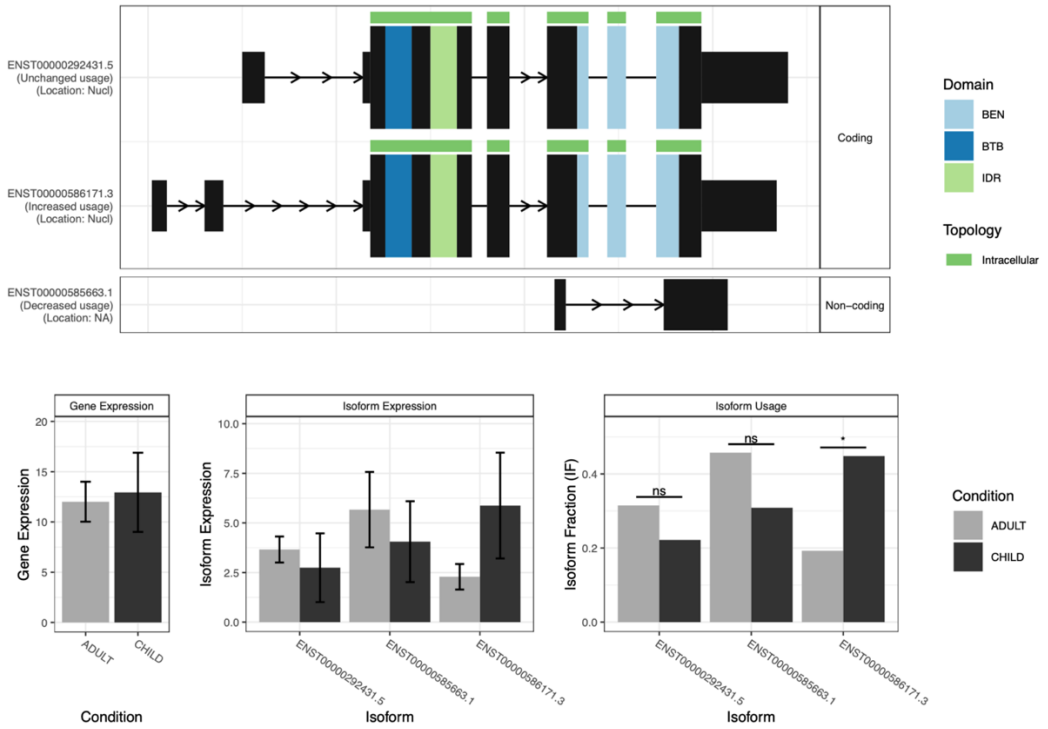


Figure 30: The isoform switch in NACC1 gene using the switchPlot functionality.

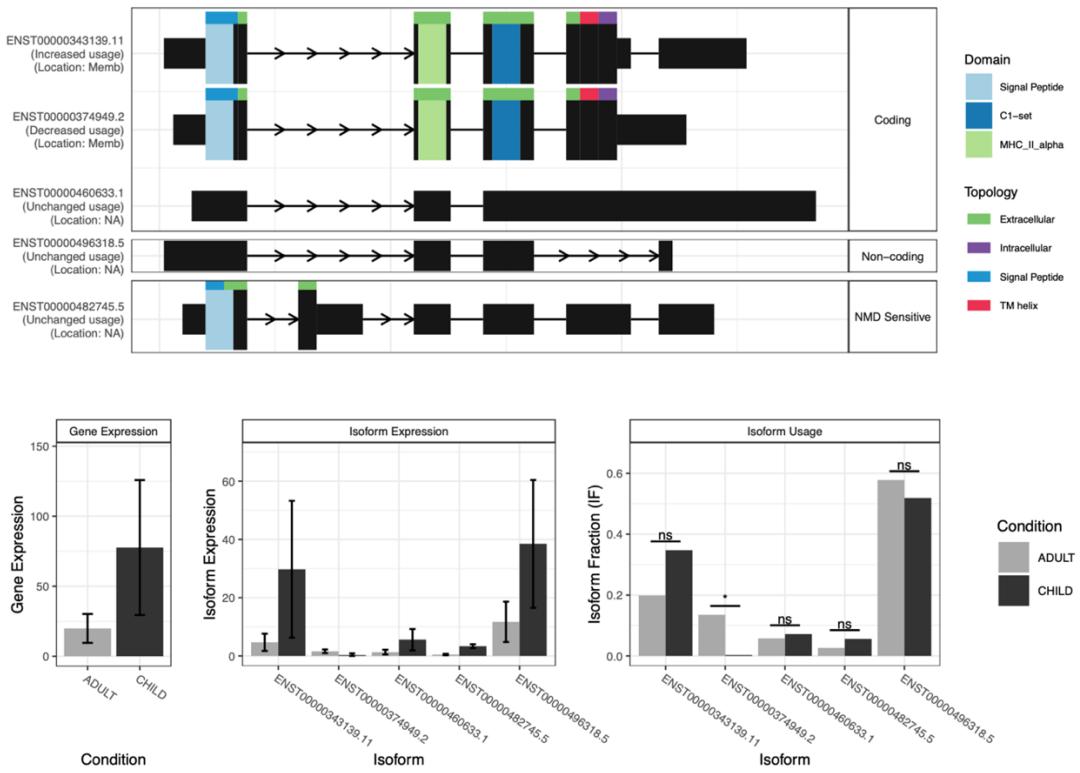


Figure 31: The isoform switch in the HLA-DQA1 gene using the switchPlot functionality.

4.6 Transcriptome data of GLUT1-DS children in pre- versus post-KD

In this section we explored the transcriptional differences and isoform switches occurring between 3 GLUT1-DS pediatric patients before the KD treatment and 12 children underwent KD to define what molecular mechanisms are implicated in KD treatment and how they improve most clinical symptoms associated with the disease.
In this experimental condition analyzed, a whole characterization of the expression profile by RNA-sequencing, along with the identification of deregulated pathways, and analysis of isoform switching, was performed.

4.6.1 Gene expression profiling

PCA and heatmap, displayed in *Figure 32a-b*, are adopted to reduce the complexity of whole transcriptome data obtained through the analysis. These plots clearly indicated differences between the two conditions, suggesting that the KD might strongly impact gene expression in GLUT1-DS pediatric patients.
Specifically, PCA visualization showed that the samples per each condition appeared separated and grouped together, indicating a similarity amongst pre-KD (green circle) and post-KD (light blue circle), but relevant differences between the two groups.
Hierarchical clustering heatmap plot showed the 60 transcripts most deregulated in pre- versus post-KD through a color code, where in green were represented the up-regulated genes and in red the down-regulated ones. Based on the differential gene expression ($|\log2FC| \geq 1$ and $FDR \leq 0.1$), clustering analysis reported in the top part of the graph revealed that pre-KD infants (n=3; colored in pink) were grouped together and separately from post-KD children (n=12; colored in light blue).
The distribution of up- and down-regulated genes was shown in a volcano plot (*Figure 32c*). Considering a total of 25.209 genes, non-differentially expressed genes were represented in grey, genes that respected only one condition in terms of log2FC and FDR were represented in blue, while genes that respected both the conditions were reported in red. Already from the graph it is possible to appreciate how there is a majority of up-regulated genes.

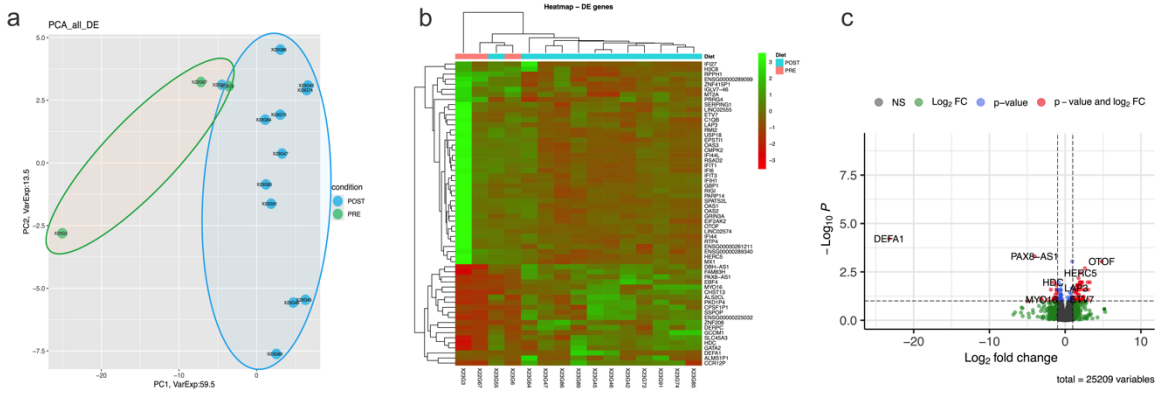


Figure 32: Transcriptome profiles in GLUT1-DS patient's PBMCs of pre-KD and post-KD. a) PCA of differently expressed genes in pre- versus post-KD; b) Expression profiles of differently expressed genes in pre- versus post-KD reported as a Heatmap; c) Volcano plot showing deregulated genes between pre- and post-KD. On the x axis is reported the log2FC whereas on the y axis is reported the P value in logarithmic scale.

4.6.1.1 Coding and non-coding biotypes

A total of 69 deregulated genes with |log2FC| ≥ 1 are detected in pre- versus post-KD comparison and reported in *Table 13*. Of these, 53 were protein coding genes (mRNAs; 41 up-regulated and 12 down-regulated), 10 were non-coding genes (ncRNAs; 7 up-regulated and 3 down-regulated) and 6 were pseudogenes (2 up-regulated and 4 down-regulated).
The subtype characterization of the ncRNAs is reported in *Table 14* with a classification of these ncRNAs for their specific biotype. It is possible to note how the most abundant category was long non-coding RNA (lncRNA) detailed in section 4.6.4.

	mRNAs	**ncRNAs**	**Pseudogenes**	**Total**
Upregulated	41	7	2	**50**
Downregulated	12	3	4	**19**
Total	**53**	**10**	**6**	**69**

Table 11: Number of differentially expressed RNAs in pre- versus post-KD comparison.

	lncRNAs	**snoRNAs**	**RNase_P_RNA**	**Total**
Upregulated	5	1	1	**7**
Downregulated	3	0	0	**3**
Total	**8**	**1**	**1**	**10**

Table 12: Biotype characterization of differentially expressed ncRNAs.

The most 20 deregulated genes (10 up-regulated and 10 down-regulated) based on their log2FC are reported in *Table 15.* Among the deregulated genes it is possible to highlight an implication for genes involved in interferon signaling and innate immune response such as *IFIT1*, *IFIH1* and *RSAD2*, upregulated in pre-KD.

Moreover, STRING tool was used to define an interaction network of DEGs where the nodes are proteins, and the edges represent the predicted functional associations (*Figure 33*). It is possible to see that the proteins encoded by these genes interact in one main network, with evidence which is both from curated databases and experimentally determined.

Transcript_ID	**gene_name**	**log2FC**	**padj**	**Category**
ENSG00000115155	OTOF	4,8202207	0,00091124	Protein coding
ENSG00000137959	IFI44L	3,26243386	0,01089135	Protein coding
ENSG00000173369	C1QB	3,06124486	0,02611160	Protein coding
ENSG00000290727	ALMS1P1	3,03162705	0,08769971	Pseudogene
ENSG00000134321	RSAD2	2,9663973	0,01089135	Protein coding
ENSG00000137965	IFI44	2,65269802	0,02612674	Protein coding
ENSG00000185745	IFIT1	2,6053917	0,00205991	Protein coding
ENSG00000149131	SERPING1	2,54145172	0,09905445	Protein coding
ENSG00000198785	GRIN3A	2,43826753	0,06089021	Protein coding
ENSG00000277209	RPPH1	2,38524238	0,06486924	RNase_P_RNA
ENSG00000180767	CHST13	-1,5431841	0,08309010	Protein coding
ENSG00000140287	HDC	-1,5945384	0,01089136	Protein coding
ENSG00000088881	EBF4	-1,6473886	0,09220063	Protein coding
ENSG00000137878	GCOM1	-1,8889983	0,02611160	Protein coding
ENSG00000160321	ZNF208	-2,3535755	0,09276935	Protein coding
ENSG00000041515	MYO16	-3,1151507	0,08432477	Protein coding
ENSG00000238241	CCR12P	-3,1728709	0,07765341	Pseudogene
ENSG00000189223	PAX8-AS1	-4,0307881	0,00049497	lncRNA
ENSG00000214076	CPSF1P1	-4,914205	0,09905445	Pseudogene
ENSG00000206047	DEFA1	-23,243127	0,00006095	Protein coding

Table 13: FC of top 20 DE RNAs. Log2FC and padj are shown for each transcript.

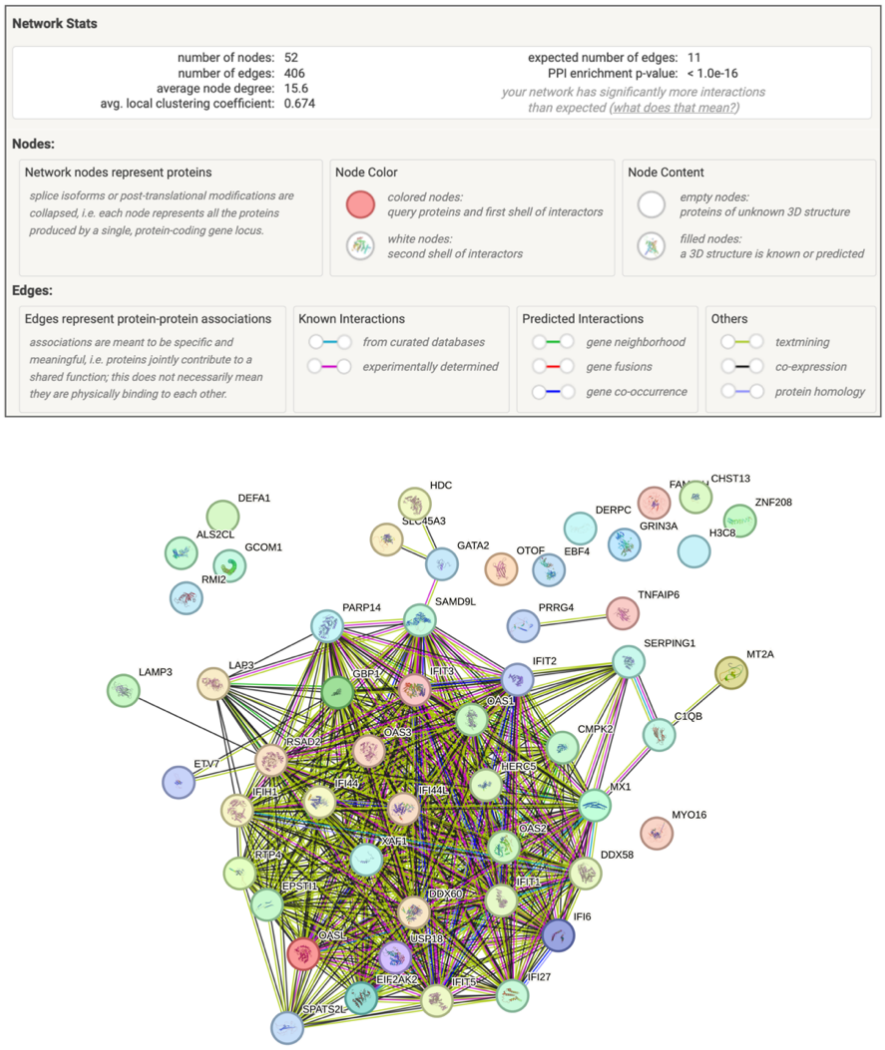


Figure 33: STRING PPI network for DEGs detected in pre- versus post-KD comparison.

4.6.2 Gene Ontology terms enrichment: Cellular Components, Molecular Functions and Biological Processes

Deregulated protein coding genes resulting from comparison were then analyzed for Gene Ontology (GO) terms enrichment, to gain insight into Cellular Components (CC), Molecular Functions (MF) and Biological Processes (BP).

The GO terms analysis in CC highlighted 18 pathways. Interestingly, adaptive immune response is amongst the most overrepresented biological terms (*Figure 34a*). The GO terms analysis for BP highlighted 53 deregulated pathways. The top GO:BP supported the observation made in the CC analysis, as half of the terms relate to processes related immune-related functions (*Figure 34b*).

The GO terms analysis in MF highlighted 41 pathways and the emapplot in *Figure 35* showed the top deregulated processes according to their significance. Interestingly, voltage-

gated ion channel (GO:0022832) and passive transmembrane transporter activities (GO:0022803) are amongst the most overrepresented terms.

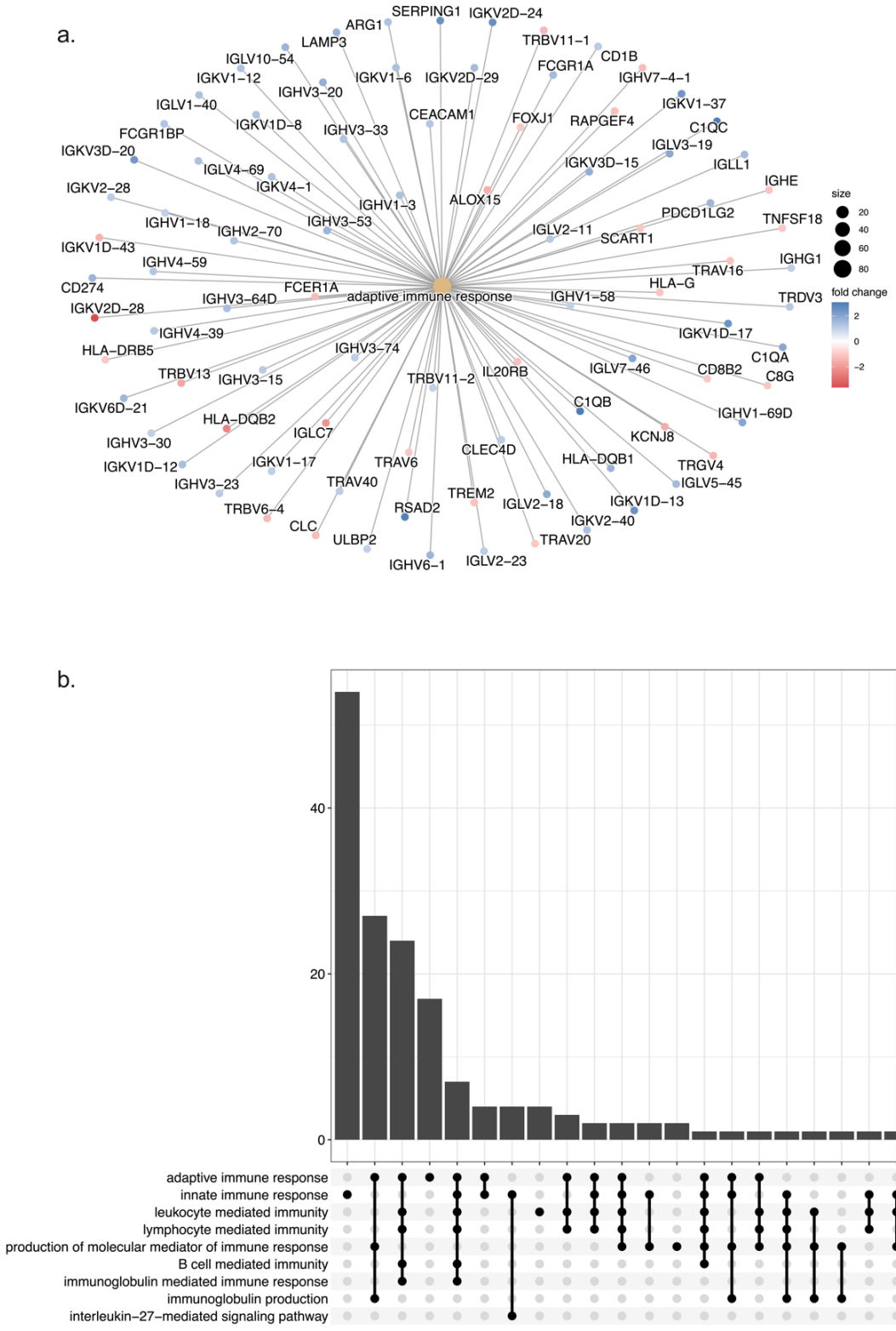


Figure 34: a) Cnetplot for GO:CC analysis depicts the linkages of genes and biological term "adaptive immune response" as network; b) Upsetplot for GO:BP analysis emphasizes the gene overlapping among different gene sets.

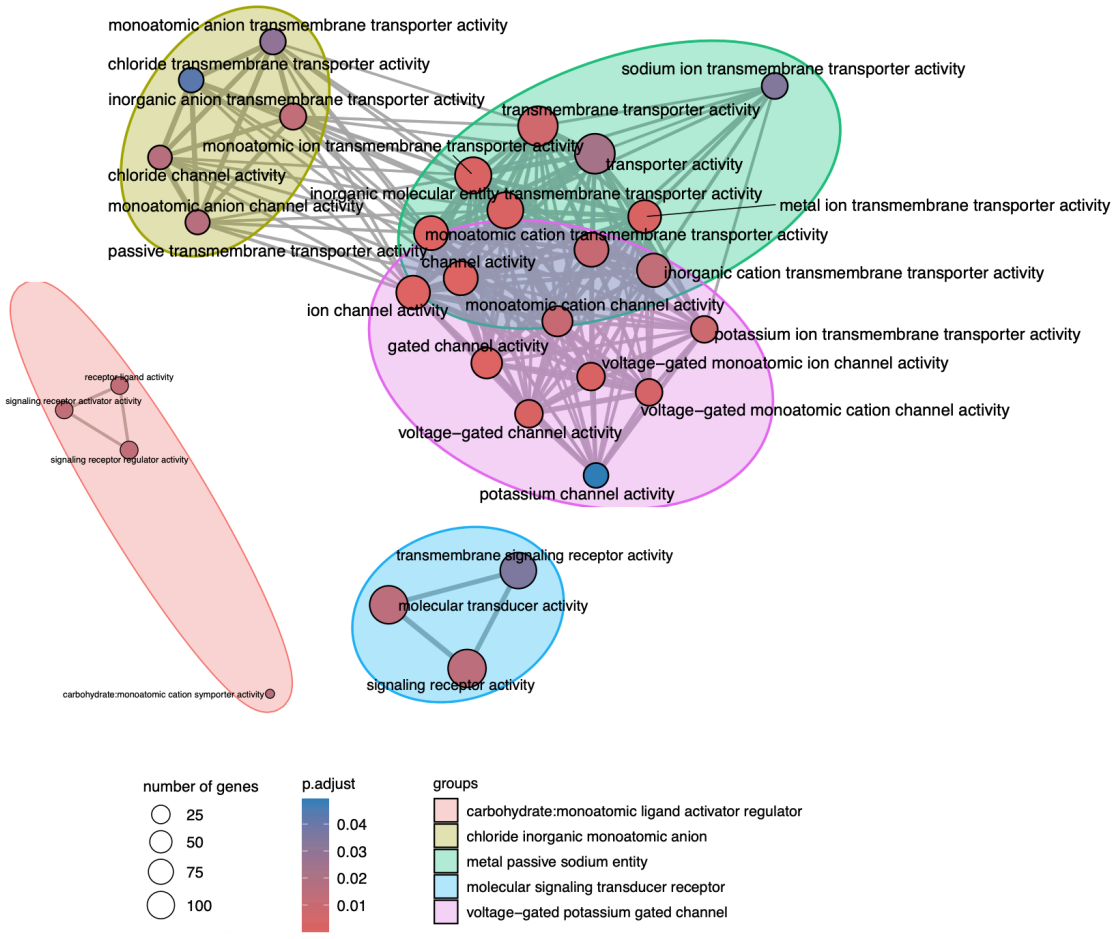


Figure 35: Emapplot for GO:MF analysis describes gene sets as an enrichment map.

4.6.3 Pathway analyses: KEGG, WikiPathways and Reactome

KEGG, WikiPathways and Reactome analyses were performed using GSEA to evaluate global gene expression changes into biological pathways. All analyses highlighted interferon signalling among the significantly deregulated processes, supporting the GO analyses.

The deregulated pathways ranked for their significance are reported for both ORA and GSEA Reactome analyses in Treeplot (*Figure 36a*) and Ridgeplot (*Figure 36b*), respectively. A strong epigenetic regulation component is represented, with DNA methylation, histone modification and various RNA-mediated processes, many more found deregulated. Moreover, mRNA Splicing (hsa03040) and mRNA processing pathways via Spliceosome resulted upregulated in pre-KD patients by KEGG analysis, suggesting an involvement of RNA splicing process (Cnetplot in *Figure 37* showed the involved genes) in dietary treatment.

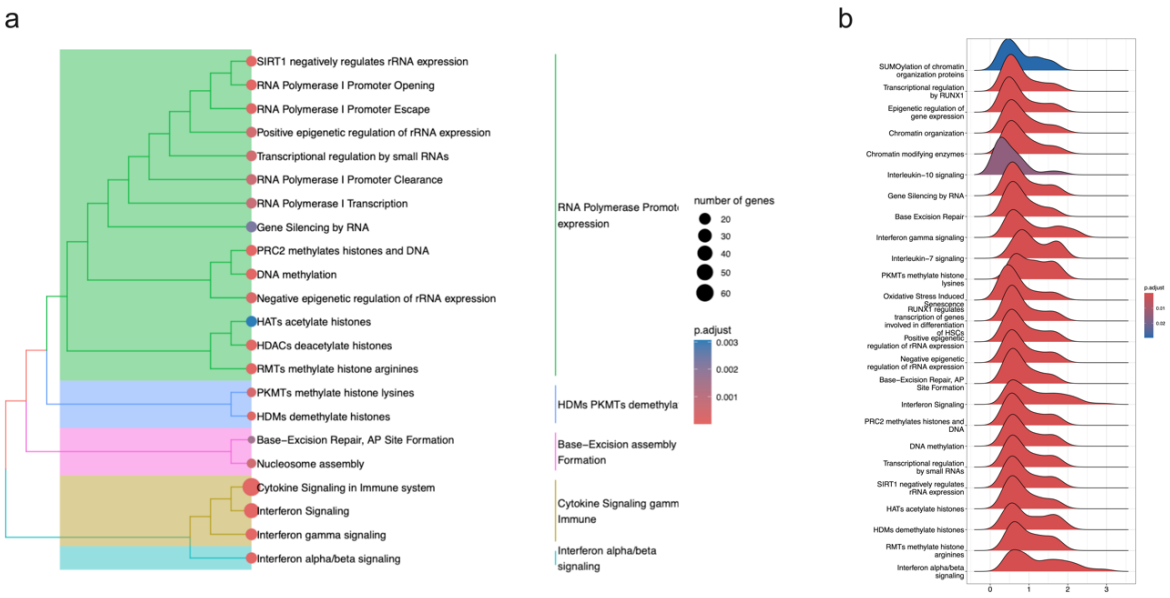


Figure 36: Reactome enrichment analysis. a) Treeplot for ORA Reactome analysis depicts the hierarchical clustering of enriched terms; b) Ridgeplot for GSEA Reactome analysis; the y-axis represents the name of the pathway, the x-axis represents the log2FC, the shape describes the density/number of genes with a specific log2FC value, and the color indicates the adjusted p-adjust as listed in the color bar.

Biological Process (Gene Ontology)

GO-term	description
GO:0000389	mRNA 3-splice site recognition
GO:0000375	RNA splicing, via transesterification reactions
GO:0000245	Spliceosomal complex assembly
GO:0000398	mRNA splicing, via spliceosome
GO:0006397	mRNA processing

Cellular Component (Gene Ontology)

GO-term	description
GO:0071014	post-mRNA release spliceosomal complex
GO:0000974	Prp19 complex
GO:0005685	U1 snRNP
GO:0071007	U2-type catalytic step 2 spliceosome
GO:0071013	Catalytic step 2 spliceosome

Reactome Pathways

pathway	description
HSA-72163	mRNA Splicing - Major Pathway
HSA-8953854	Metabolism of RNA

KEGG Pathways

pathway	description
hsa03040	Spliceosome

Annotated Keywords (UniProt)

keyword	description
KW-0747	Spliceosome
KW-0508	mRNA splicing

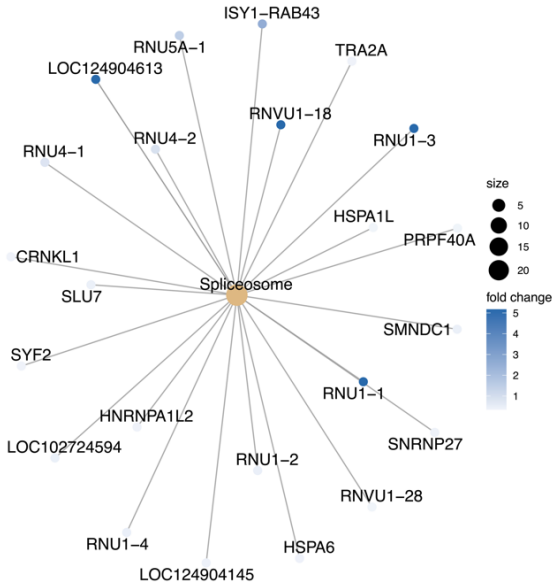


Figure 37: Cnetplot for KEGG analysis shows the linkages of genes and biological term "spliceosome" as network.

4.6.4 Computational and functional predictions of snoRNA and lncRNAs

The snoRNA, SCARNA12, was upregulated in GLUT1-DS patients before KD. It is predicted to guide post-transcriptional modification (both 2-prime-O-methylation and pseudouridylation) of RNAs (Darzacq et al., 2002).

When focusing on lncRNAs, the comparison revealed 5 up-regulated lncRNAs (LINC02555, LINC02574, ENSG00000289099, ENSG00000261211 and ENSG00000289340) and 3 downregulated lncRNAs (PAX8-AS1, DBH-AS1 and ENSG00000225032), reported in *Table 16*. The lncRNA-gene co-expression analysis was also performed using the lncHub2, providing predictions on biological functions and secondary structures of 6 out of 8 detected lncRNA (for two of these no data have been reported).

Focusing on BP, of particular interest is their involvement in biological pathways detected in coding transcriptome analyses, e.g., the regulation of oxidative phosphorylation, the regulation of innate immune response, the ion transport, the regulation of cation channel activity, and the regulation of ketone biosynthetic process. KEGG analysis revealed the synthesis and degradation of ketone bodies, the fatty acid degradation, and the spliceosome as predicted biological pathways.

In *Figure 38* are reported secondary structure predicted with RNAfold.

Transcript_ID	**gene_name**	**log2FC**	**padj**
ENSG00000260943	LINC02555	2,2484070	0,07535989
ENSG00000233975	LINC02574	1,9498211	0,09938119
ENSG00000289099	ENSG00000289099	1,8962283	0,02691342
ENSG00000261211	ENSG00000261211	1,7490131	0,07905118
ENSG00000289340	ENSG00000289340	1,0441403	0,06486924
ENSG00000225032	ENSG00000225032	-1,1012619	0,07535989
ENSG00000225756	DBH-AS1	-1,1406431	0,07765341
ENSG00000189223	PAX8-AS1	-4,0307881	0,00049497

Table 14: List of lncRNAs in pre- versus post-KD. Log2FC and padj are indicated for each transcript.

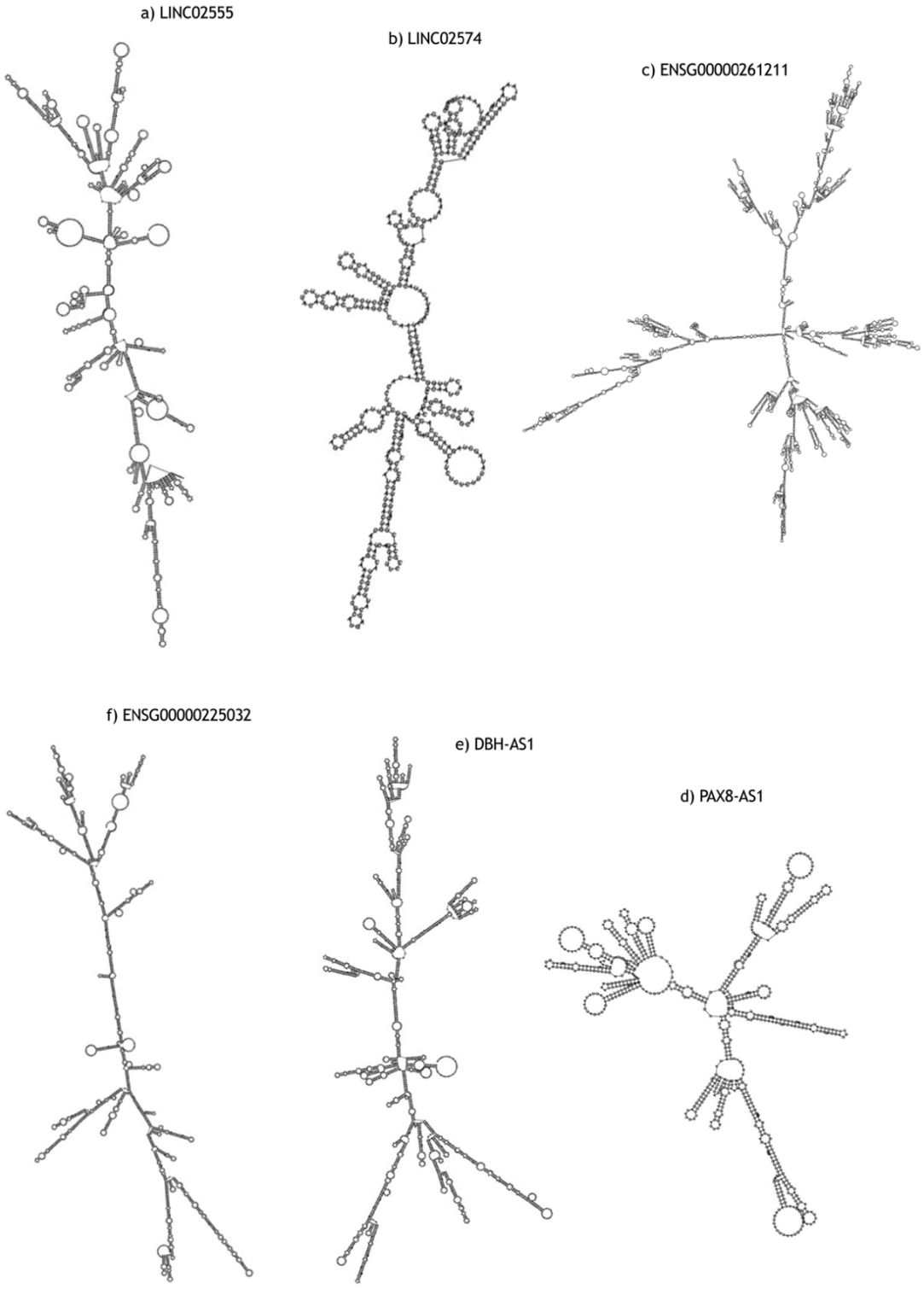


Figure 38: Predicted secondary structure of lncRNAs as obtained RNA fold web server.

4.6.4.1 Comparison between lncRNA-correlated genes and DEGs

The top 25 genes positively and negatively correlated with the identified lncRNA have been compared with 69 DEGs detected pre- versus post-KD comparison. This highlighted 4

upregulated coding protein genes, including *RSAD2, IFIT3, HERC5* and *SAMD9L*, which showed a positive correlation with upregulated lncRNA LINC02555. Interestingly, all the genes are involved in innate immune response, cytokine signaling and interferon alpha/beta signaling.

4.6.5 Identification of isoforms switching events

To further investigate the splicing dysregulation detected in GLUT1-DS children in pre- versus post-KD comparison, we analyzed variations in splicing events and isoform levels. The differential isoform expression analysis highlighted 325 differentially expressed isoforms with switching features associated to 281 unique genes which were mostly protein coding genes *(Table 17*). Given the abundance of isoforms identified with the above analysis, we first explored the molecular mechanisms underlying these changes through the consequence enrichment splicing analysis and for each type of splicing, the total number of events detected. The switched isoforms are involved in multiple alternative splicing (AS) events, noting that the AS events are not equally used. As reported in *Figure 39a,* when considering the number of AS events, ATSS and ATTS are the most predominant with 728 and 670 events respectively, followed by alternative 5′ donor sites (408 A5 events), alternative 3′ acceptor sites (405 A3 events), exon skipping (389 ES events), multiple exon skipping (220 MES events), IR (132 events) and mutually exclusive exons (MEE) with only 6 events. When considering opposite consequence analysis (e.g. the gain versus loss of ATSS) the patterns looked mostly similar, as shown in *Figure 39b.*

Once we analyzed the mechanisms underlying the changes observed, we focused our attention on the different isoform switches highlighted by the differential analysis. As shown in *Figure 39c*, the most frequent changes are the ones affecting protein domains (214 events), coding potential (180 events), intrinsically disordered regions (126 IDR events) and intron retention (88 events). Although it displayed a similar distribution of impacts, we deeply and systematically investigated for all consequences using the built-in enrichment analysis and reported in *Figure 39d.* For each pair of opposite consequences (e.g. protein domain loss vs gain and so on) reported on the y-axis, the analysis highlights the fraction of switches, affected by either of the opposing consequence, that results in the consequence indicated (e.g., protein domain loss) reported in x-axis. If the fraction is significantly different from 0.5, there is a systematic bias in isoform switch consequences. According to this analysis, all the opposing consequences are evenly distributed, suggesting that there were no differences in children with GLUT1-DS before KD.

ZBTB14	MTRF1	BORCS8-MEF2B	RAD50	SLC25A15
DDX41	TREML1	CDKL1	NUDCD1	ZFP1
TBCA	ZNF250	ELOVL7	TMEM63B	USP45
NDUFA7	GZF1	CEACAM1	TNFRSF1B	NFKBIL1
CPVL	ATG4D	MPDU1	ZBED1	C11orf68
PHACTR2	BRD3	SLAIN1	AKAP1	UPRT
PRF1	VCPKMT	GHRL	TSFM	DAAM1
PISD	PTPMT1	CCDC9	NECTIN1	TUBA8
DHX8	IGLL5	GNL2	SIRPD	PTK2
SPIN2B	BTBD9	SIGMAR1	CMAHP	RNF8
GGA3	HYI	PGAP2	XYLT1	ZC3HC1
MYG1	ENSG00000238009	ODF2	CYREN	C17orf80
METTL26	CPM	STK19	SIRT6	LINC01278
LIG4	CLDN12	PKD1	ACY1	SMAGP
AGER	MCM9	ABL1	HLCS	AKIP1
CD33	SCFD2	VPS72	GRSF1	GPR132
WWP1	PMPCA	G2E3	CDK6	POLD2
LPIN1	TARBP2	RRP8	RWDD4	SLC25A45
PPP1R7	NTAN1	NAE1	WDR41	DHFR
AKAP7	KAT5	CHM	PWWP2B	ZEB1-AS1
AFG3L2	HLX	TVP23B	TARS1	ZNF839
RELA-DT	ENSG00000225889	7,00 LSM	TAF1C	PLEK2
CSNK1G3	PCCA	ZNF559	LINC00539	ZNF324
ZDHHC3	ZNF691	KDM8	NSF	GMPPA
FFAR2	RFK	SLC35B2	ZNF419	PDE5A
RIOK1	MRPL9	MYLK	SLC39A13	ZNF630
WIPI1	PIN4	ACBD4	RGPD5	PDCD11
GTF2B	RAD17	DDIT4	IFNAR2-IL10RB	SLC39A8
SLC29A1	GPD1L	U2AF1L4	NUDT7	NTNG2
MIX23	ZNF702P	TMEM147-AS1	NICN1	KIFC3
SPINT2	BATF3	MLEC	SLC35B3	P2RX7
STK26	SCMH1	TGFBR3	DPY19L1	RABEPK
ZNF282	TRMT2A	HDDC2	DPAGT1	ZNF544
SMANTIS	WDR25	NAPRT	PRKAG2-AS1	FBXO25
ZNF626	PIAS4	ZNF687	FPGT	CES1
SEPTIN11	PLAAT3	SPATA24	FAM50B	CCNQ
THOC6	VSTM1	BCL9L	TMEM126B	TRAPPC6A
EFCAB2	CAPN12	CRADD	ANAPC10	DGKH
TFIP11	FHL1	NDUFS1	SUMF1	RPL23AP7
KEAP1	USP13	MUTYH	ATF2	SGK3
DKC1	1 CLP	ZPR1	ISYNA1	NUBP2
C19orf48P	ALG10	ADARB1	MZT2B	MRPL28
ITPKC	MRM3	ASL	MEMO1	TRMT10A
PTPN23	GALNT12	ZNF782	ZNF790	WASHC2A
IREB2	AHSP	MPND	PRR12	P4HA1
RCCD1	ALAS1	CCDC106	ENO3	C3orf18
SLC10A3	ZBTB43	LCORL	ZNF575	ZNF510
HSD17B12	CLEC10A	ATP13A2	DGKQ	IFT57
STYXL1	HDGFL2	TSPYL2	VTI1B	ZCRB1
MFGE8	ZFP90	MGAT5	TMPO-AS1	TNFRSF12A
NELFCD	EOLA1	SYNJ1	MTIF3	CCDC149
NAT9	FCRLA	ERLEC1	LIN37	RRP1
TMEM170A	ERICH6-AS1	TATDN1	PALS2	TP53I3
RTF1	ST13	TM2D2	TRIM62	
POLR2F	SUOX	CROT	HSCB	
SNRPA	SARS2	GTF2H3	TRIM39	
SLC12A4	FBXL17	ATXN7L2	ATPAF1	

Table 15: Switching genes involved in differential isoform expression in pre- versus post-KD.

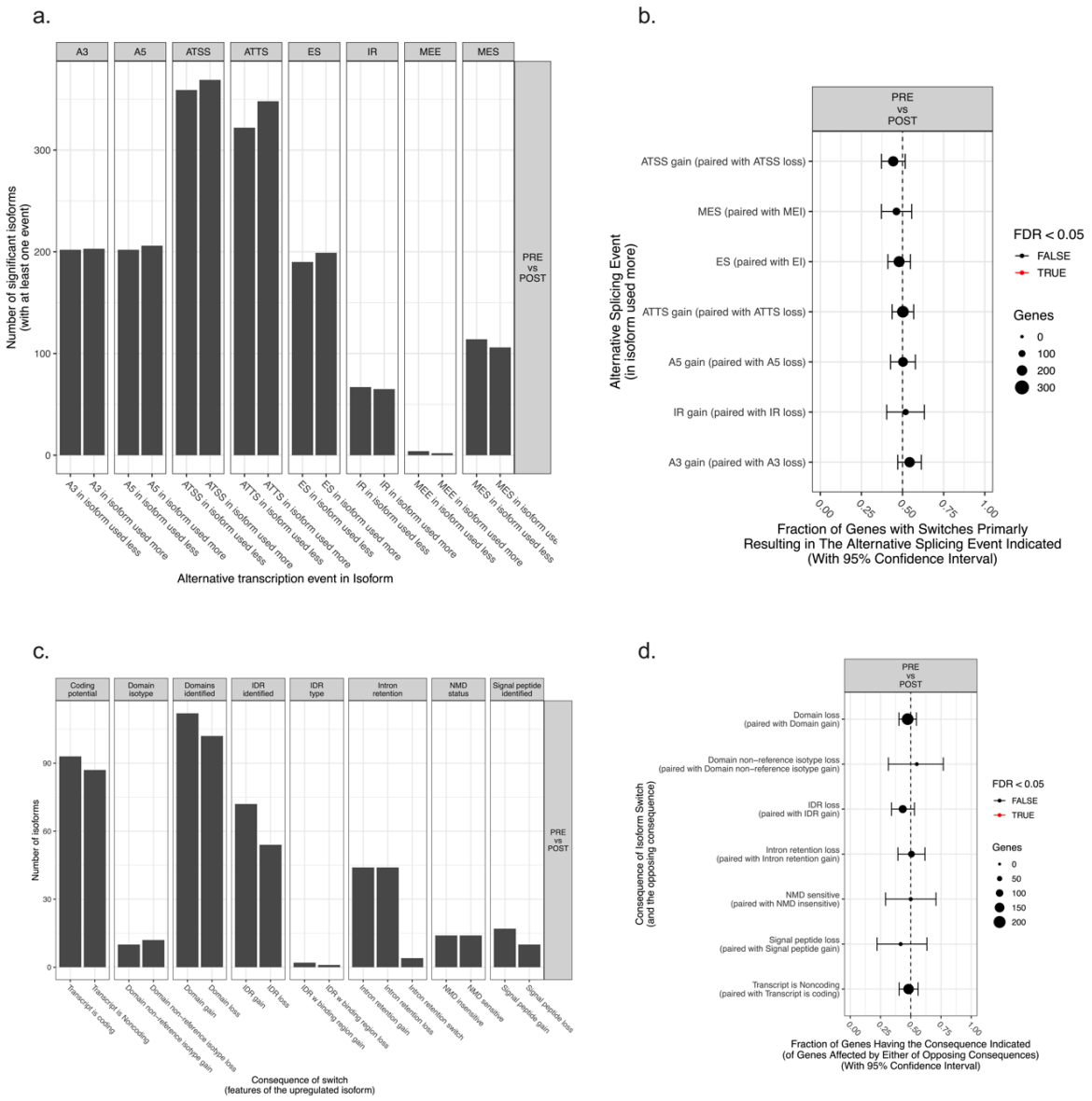
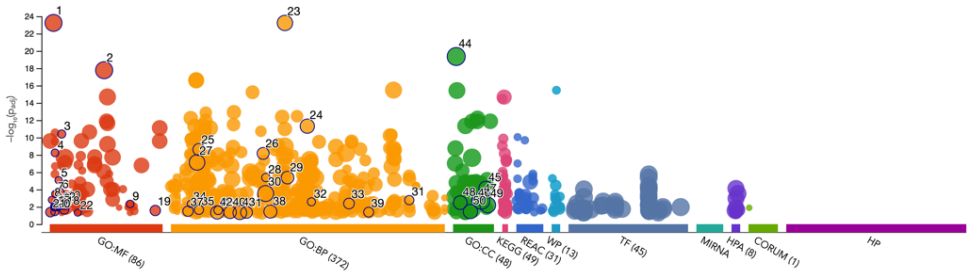


Figure 39: Transcripts analysis for switched isoforms between pre- and post-KD; a, b) Distribution of isoform usage in each type of alternative splicing; c, d) Overview of the number of switched isoforms predicted to have functional consequences.

4.6.5.1 Biological implications of switching genes

Given the evidence in alternative splicing and switching isoform analysis, we performed a functional enrichment analysis on all switching genes involved in differential isoform expression to define molecular mechanisms at the basis of this dysregulation. The analysis performed on g:Profiler webtool returned a total of 64 pathways shown in *Figure 40.* Interestingly, these pathways include regulation mechanisms such as miRNA biogenesis and gene expression, as well as metabolic and mitochondrial processes.



ID	Source	Term ID	Term Name	p_{adj} (query_...
1	GO:MF	GO:0003824	catalytic activity	5.823×10^{-24}
2	GO:MF	GO:0036094	small molecule binding	1.675×10^{-18}
3	GO:MF	GO:0004935	adrenergic receptor activity	3.917×10^{-11}
4	GO:MF	GO:0004024	alcohol dehydrogenase activity, zinc-dependent	5.840×10^{-9}
5	GO:MF	GO:0004556	alpha-amylase activity	8.441×10^{-6}
6	GO:MF	GO:0004675	transmembrane receptor protein serine/threoni...	2.228×10^{-4}
7	GO:MF	GO:0004035	alkaline phosphatase activity	3.200×10^{-4}
8	GO:MF	GO:0001609	G protein-coupled adenosine receptor activity	1.566×10^{-3}
9	GO:MF	GO:0051379	epinephrine binding	4.600×10^{-3}
10	GO:MF	GO:0005544	calcium-dependent phospholipid binding	5.327×10^{-3}
11	GO:MF	GO:0004859	phospholipase inhibitor activity	5.726×10^{-3}
12	GO:MF	GO:0004745	NAD-retinol dehydrogenase activity	6.267×10^{-3}
13	GO:MF	GO:0003995	acyl-CoA dehydrogenase activity	9.101×10^{-3}
14	GO:MF	GO:0003876	AMP deaminase activity	1.211×10^{-2}
15	GO:MF	GO:0004332	fructose-bisphosphate aldolase activity	1.211×10^{-2}
16	GO:MF	GO:0003994	aconitate hydratase activity	1.211×10^{-2}
17	GO:MF	GO:0004060	arylamine N-acetyltransferase activity	1.211×10^{-2}
18	GO:MF	GO:0005346	purine ribonucleotide transmembrane transpor...	1.988×10^{-2}
19	GO:MF	GO:0140359	ABC-type transporter activity	2.645×10^{-2}
20	GO:MF	GO:0003993	acid phosphatase activity	3.625×10^{-2}
21	GO:MF	GO:0000295	adenine nucleotide transmembrane transporte...	4.086×10^{-2}
22	GO:MF	GO:0016401	palmitoyl-CoA oxidase activity	4.749×10^{-2}
23	GO:BP	GO:0044281	small molecule metabolic process	5.855×10^{-24}
24	GO:BP	GO:0048871	multicellular organismal-level homeostasis	4.858×10^{-12}
25	GO:BP	GO:0007189	adenylate cyclase-activating G protein-couple...	2.423×10^{-9}
26	GO:BP	GO:0035150	regulation of tube size	6.784×10^{-9}
27	GO:BP	GO:0006915	apoptotic process	7.469×10^{-8}
28	GO:BP	GO:0035588	G protein-coupled purinergic receptor signalin...	4.110×10^{-6}
29	GO:BP	GO:0044843	cell cycle G1/S phase transition	4.231×10^{-6}
30	GO:BP	GO:0035556	intracellular signal transduction	2.890×10^{-4}
31	GO:BP	GO:1903523	negative regulation of blood circulation	1.676×10^{-3}
32	GO:BP	GO:0051122	hepoxilin biosynthetic process	2.631×10^{-3}
33	GO:BP	GO:0072132	mesenchyme morphogenesis	4.148×10^{-3}
34	GO:BP	GO:0006101	citrate metabolic process	4.529×10^{-3}
35	GO:BP	GO:0007190	activation of adenylate cyclase activity	2.195×10^{-2}
36	GO:BP	GO:0014061	regulation of norepinephrine secretion	2.475×10^{-2}
37	GO:BP	GO:0003254	regulation of membrane depolarization	3.189×10^{-2}
38	GO:BP	GO:0036293	response to decreased oxygen levels	3.540×10^{-2}
39	GO:BP	GO:0099171	presynaptic modulation of chemical synaptic tr...	4.088×10^{-2}
40	GO:BP	GO:0019318	hexose metabolic process	4.270×10^{-2}
41	GO:BP	GO:0031623	receptor internalization	4.413×10^{-2}
42	GO:BP	GO:0010959	regulation of metal ion transport	4.513×10^{-2}
43	GO:BP	GO:0022411	cellular component disassembly	4.863×10^{-2}
44	GO:CC	GO:0005737	cytoplasm	4.400×10^{-20}
45	GO:CC	GO:0098794	postsynapse	7.051×10^{-5}
46	GO:CC	GO:0048179	activin receptor complex	1.738×10^{-3}
47	GO:CC	GO:0071944	cell periphery	1.947×10^{-3}
48	GO:CC	GO:0015629	actin cytoskeleton	3.298×10^{-3}
49	GO:CC	GO:0120025	plasma membrane bounded cell projection	6.270×10^{-3}
50	GO:CC	GO:0036477	somatodendritic compartment	3.503×10^{-2}

version	e111_eg58_p18_f463989d
date	31/10/2024, 15:00:22
organism	hsapiens

g:Profiler

Figure 40: Enrichment analysis for GO on switching genes. The upper graph represents the number of terms involved for each biological analysis, the table shows ID and name of the pathways.

Additionally, analysis for Human Phenotype Ontology (HPO) highlighted terms such as acute encephalopathy (HP:0006846), hypoproteinemia (HP:0003075), abnormality of sodium homeostasis (HP:0010931), and others reported in *Figure 41*, confirming the findings underlined by the differential expression analysis.

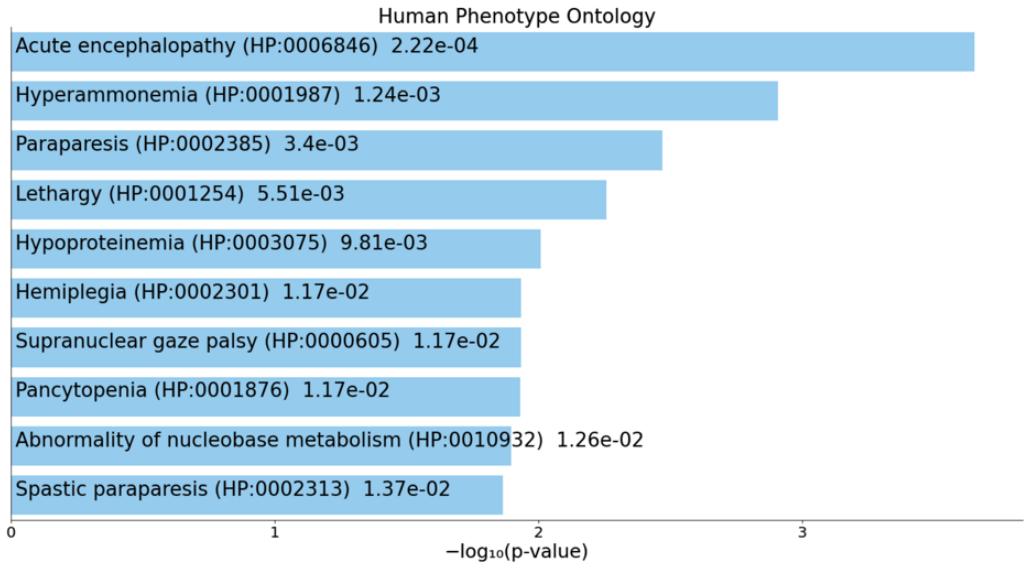


Figure 41: Analysis for HPO terms.

4.6.5.2 Significant alternative splicing isoforms

Two parallel investigations were performed: (1) a bibliographic analysis using PubMed source, to identify how many, amongst the 281 genes had been associated with keywords as GLUT1-DS and/or epilepsy, and (2) a comparison between these 281 genes affecting alternative splicing and ion channels data (69 genes). These analyses revealed that only one switching gene, *P2X7*, was shared between both studies.

P2X7 gene (OMIM #602566; 12q24.31) encodes a gated-ion channel that mediates Na^+ and Ca^{2+} influx and K^+ efflux. Its activation occurs under pathological conditions of high ATP release, for example during inflammation or increased neuronal activity (e.g. seizure). Consequently, this leads to upregulation of the GLUT1 via the PI3K-AKT pathway and promotes both glycolysis and mitochondrial respiration (De Salles et al., 2023; Engel, 2023).

The isoform switch analysis for this gene illustrated in *Figure 42* showed no difference in the gene expression of P2RX7 between the two conditions, but a significant switch in isoform usage across conditions is manifest. Five isoforms derived from alternative splicing are revealed: one full-length coding (P2RX7-202; transcript ENST00000328963), two non-coding (ENST00000535928.5, ENST00000545434.5) and two nonsense-mediated RNA decay (NMD) sensitive (ENST00000535250.5, ENST00000541716.5) transcripts.

By comparing which isoforms are changing to the isoform structure, it can be inferred that in the post-KD samples it is the short non-coding transcript (P2RX7-J/213; transcript ENST00000545434.5) that is used. Schematic features of both canonical, P2RX7-202, and non-coding, P2RX7-J, transcripts are displayed in *Figure 43*.

We therefore reported a different gene expression of detected isoforms in pre- versus post-KD. In detail, the canonical transcript is downregulated in post-KD, where resulted

expressed the P2X7-J non-coding isoform. KD might regulate a P2X7 isoform switch by an alternative splicing mechanism.

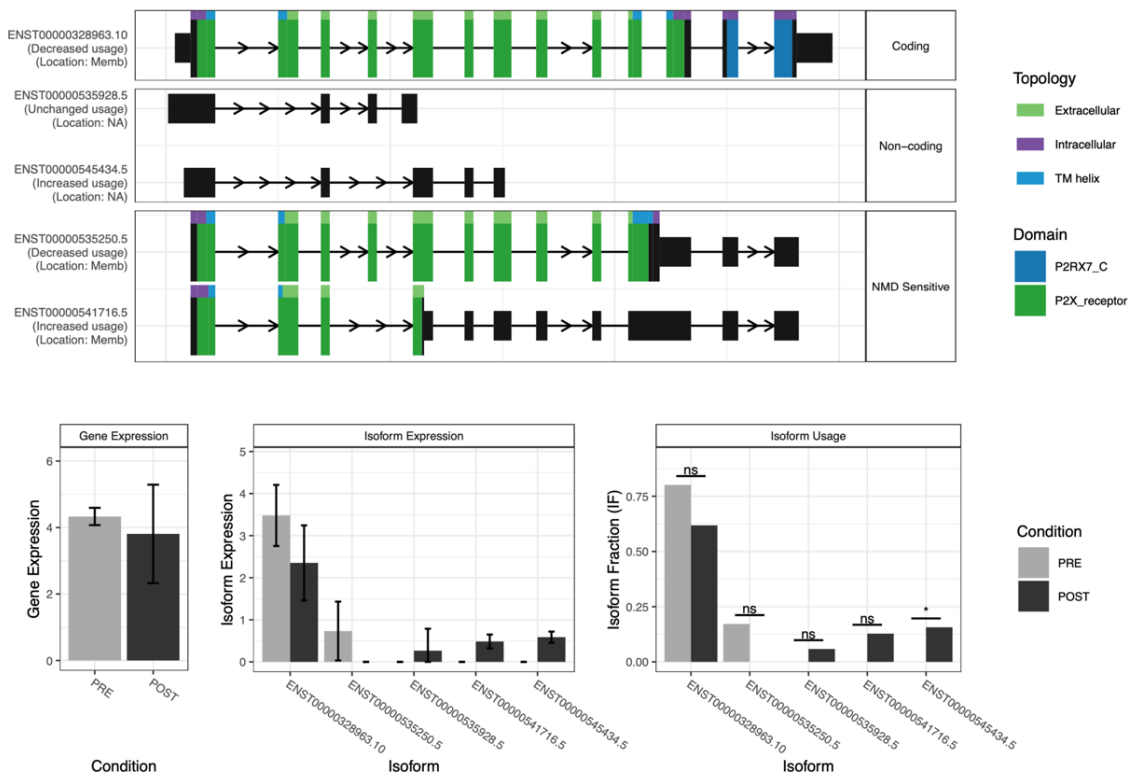


Figure 42: The isoform switch in the P2RX7 gene using the switchPlot functionality.

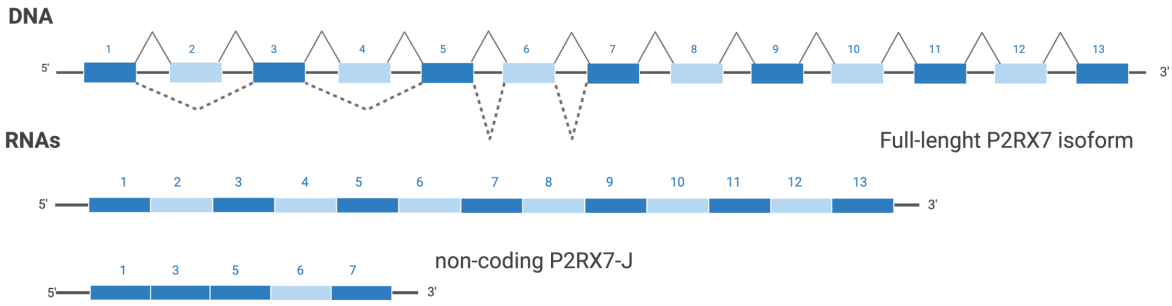


Figure 43: Alternative transcript processing generates isoform diversity. Splice junctions demonstrate inclusion or exclusion of transcript exons (non-coding P2RX7-J) compared to constitutive exons (full-length P2RX7 isoform) (Created with BioRender).

4.6.5.3 Expression analysis in qPCR of P2X7 isoforms

P2X7 switching gene was also investigated by qPCR assay. A significative down-regulation of full-length P2RX7 coding transcript was observed in PBMCs of GLUT1-DS children who underwent KD compared to pediatric patients before diet treatment. In this same condition,

on the other hand, the non-coding P2RX7-J isoform was detected significantly up-regulated (*Figure 44*), suggesting a significantly differed RNA isoforms among the two conditions in terms of P2X7 gene.

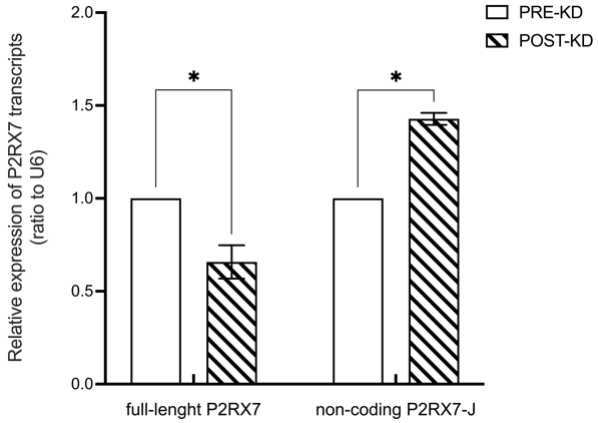


*Figure 44: Relative expression analysis of P2X7 isoforms by qPCR. U6 was used as housekeeping gene (*p<0.05).*

4.7 Transcriptome data of responders versus non-responders to KD in GLUT1-DS children

In this section we explored the molecular pathways that change when ketosis fail to improve symptoms through the transcriptional differences occurring between 2 GLUT1-DS children in KD with good clinical outcomes and 2 GLUT1-DS children in KD with bad response.
In this experimental condition, a whole characterization of the expression profile, along with the identification of deregulated pathways, was performed.

4.7.1 Gene expression profiling

PCA and heatmap, shown in *Figure 45a-b,* are adopted to reduce the complexity of whole transcriptome data obtained through the analysis. These graphs clearly indicated differences among the conditions, suggesting that gene expression in GLUT1-DS pediatric patients who underwent KD might be impacted by good/bad clinical outcomes.
Specifically, PCA plot displayed that the samples per each condition appeared separated and grouped together, indicating a similarity amongst samples with bad response to KD (light blue circle) and samples with good response to KD (yellow circle), and relevant differences between the two conditions.
Heatmap showed the 60 transcripts most deregulated through a color code, where in green were represented the up-regulated genes and in red the down-regulated ones. Based on the differential gene expression ($|\log2FC| \geq 1$ and $FDR \leq 0.1$), clustering analysis reported in the top part of the heatmap graph revealed that good response samples (n=2; colored in pink)

were grouped together and separately from bad response samples (n=2; colored in light blue).

The distribution of up- and down-regulated genes was shown in a volcano plot (*Figure 45c*). Considering a total of 21.829 genes, non-differentially expressed genes were represented in grey, genes that respected only one condition in terms of log2FC and FDR were represented in blue, while genes that respected both the conditions were reported in red. Already from the graph it is possible to appreciate how there is a majority of down-regulated genes.

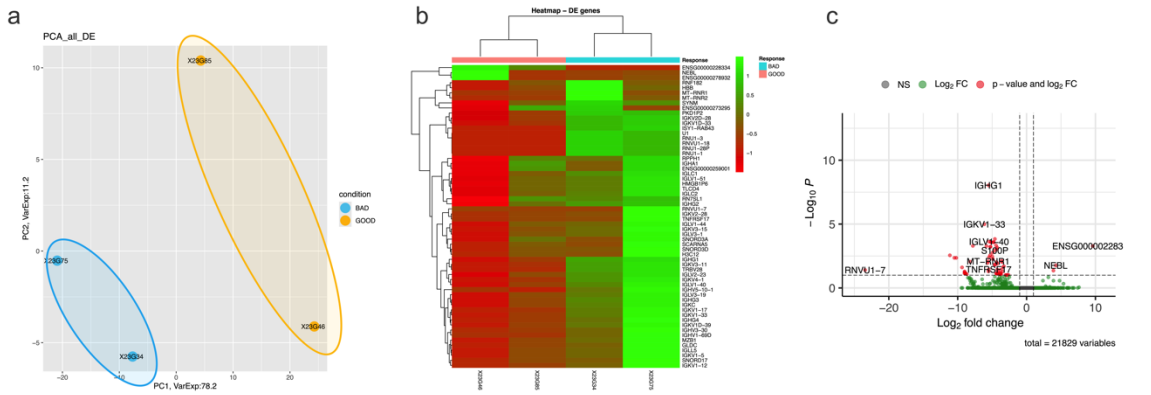


Figure 45: Transcriptome profiles in GLUT1-DS patient's PBMCs of responders and non-responders; a) PCA of differently expressed genes in good versus bad; b) Expression profiles of differently expressed genes in good versus bad reported as a Heatmap; c) Volcano plot showing deregulated genes between good and bad. On the x axis is reported the log2FC whereas on the y axis is reported the P value in logarithmic scale.

4.7.1.1 Coding and non-coding biotypes

A total of 68 deregulated genes with |log2FC| ≥ 1 are detected in responders versus non-responders comparison and reported in *Table 18*. Of these, 48 were protein coding genes (mRNAs; 1 up-regulated and 47 down-regulated), 16 were non-coding genes (ncRNAs; 2 up-regulated and 14 down-regulated) and 4 were pseudogenes (4 down-regulated).

The subtype characterization of the ncRNAs is reported in *Table 19* with a classification of these ncRNAs for their specific biotype. It is possible to note how the most abundant categories were long non-coding RNA (lncRNA), small nucleolar RNA (snoRNA), and small nuclear RNA (snRNA), detailed in section 4.7.4.

	mRNAs	**ncRNAs**	**Pseudogenes**	**Total**
Upregulated	1	2	0	**3**
Downregulated	47	14	4	**65**
Total	**48**	**16**	**4**	**68**

Table 16: Number of differentially expressed RNAs in responders versus non-responders comparison.

	lncRNAs	snoRNAs	snRNA	rRNA	SRP_RNA	RNase_P_RNA	Total
Upregulated	2	0	0	0	0	0	**2**
Downregulated	2	4	4	2	1	1	**14**
Total	**4**	**4**	**4**	**2**	**1**	**1**	**16**

Table 17: Biotype characterization of differentially expressed ncRNAs.

The most 20 deregulated genes based on their log2FC are reported in *Table 20*. Among the down-regulated genes it is possible to highlight an implication for genes involved in RNA modifications and alternative splicing, such as RNVU1-7, SNORD3D and ISY1-RAB43. Genes, like IGKV2D-28, IGKV1D-33 and IGHV1-69D, involved in immune system process, are also down-regulated in patients with a good response when compared with children with bad response.

Transcript_ID	gene_name	log2FC	padj	Category
ENSG00000206585	RNVU1-7	-23,44653	0,0396077	snRNA
ENSG00000277947	SNORD3D	-11,13843	0,0027919	snoRNA
ENSG00000273295	ENSG00000273295	-10,48966	0,0044110	lncRNA
ENSG00000227827	PKD1P2	-10,20864	0,0044110	Pseudogene
ENSG00000242534	IGKV2D-28	-9,37232	0,0246951	Protein Coding
ENSG00000178445	GLDC	-9,01787	0,0720781	Protein Coding
ENSG00000239975	IGKV1D-33	-9,00825	0,0515698	Protein Coding
ENSG00000261796	ISY1-RAB43	-9,00272	0,0591929	Protein Coding
ENSG00000206652	RNU1-1	-8,87069	0,0714872	snRNA
ENSG00000206588	RNU1-28P	-8,87069	0,0714872	Pseudogene
ENSG00000207513	RNU1-3	-8,87069	0,0714872	snRNA
ENSG00000206737	RNVU1-18	-8,87069	0,0714872	snRNA
ENSG00000275405	U1	-8,87069	0,0714872	Protein Coding
ENSG00000259781	HMGB1P6	-8,67743	0,0844435	Pseudogene
ENSG00000280411	IGHV1-69D	-8,17333	0,0088963	Protein Coding
ENSG00000180537	RNF182	-7,79986	0,0005078	Protein Coding
ENSG00000242076	IGKV1-33	-6,13613	0,0000103	Protein Coding
ENSG00000278932	ENSG00000278932	3,89456	0,0438472	lncRNA
ENSG00000078114	NEBL	4,22972	0,0172562	Protein Coding
ENSG00000228334	ENSG00000228334	9,61383	0,0005209	lncRNA

Table 18: FC of top 20 DE RNAs.

4.7.2 Gene Ontology terms enrichment: Cellular Components, Molecular Functions and Biological Processes

Deregulated protein coding genes resulting from the comparison were then analyzed for Gene Ontology (GO) terms enrichment, to gain insight into Cellular Components (CC), Molecular Functions (MF) and Biological Processes (BP).

The GO terms analysis in CC highlighted 17 pathways. The top GO:CC showed a down-regulation in immunity response and mitochondrial protein complex in GLUT1-DS patients in KD with good clinical response (*Figure 46*).

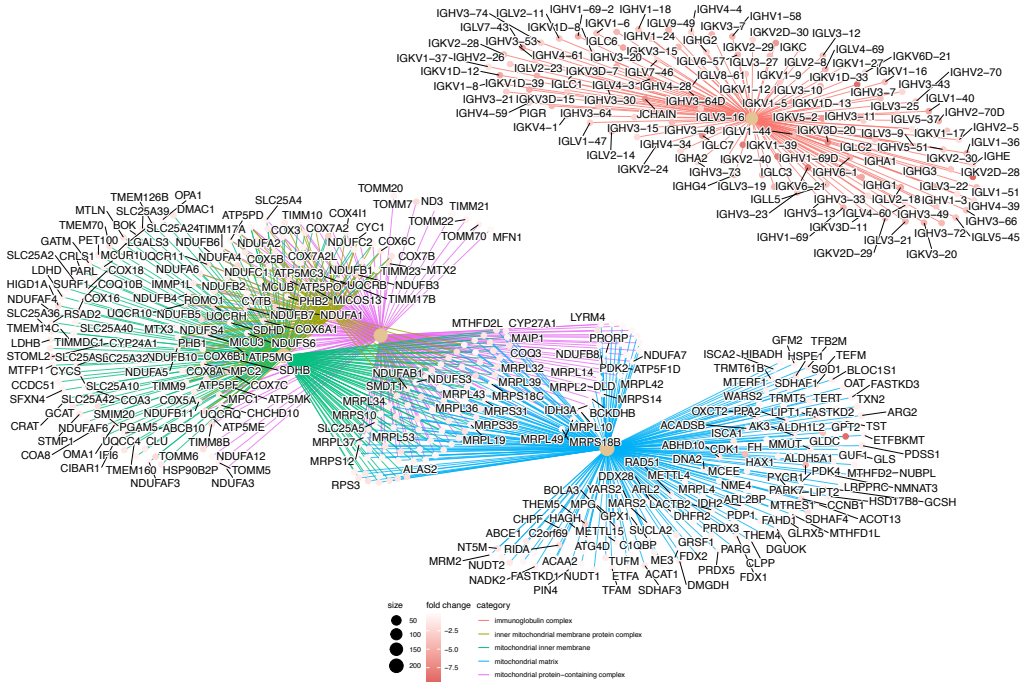


Figure 46: Cnetplot of GO:CC analysis for DE RNAs in responders versus non-responders. Data relationships are visualized in network diagrams with associated data to colour nodes.

The GO terms analysis in MF highlighted 6 pathways and the heatplot showed the top 3 deregulated processes according to their significance (*Figure 47*). Interestingly, electron transfer activity (GO:0009055) and voltage-gated channel activity (GO:0022832) are amongst the most overrepresented terms. In *Figure 48* is shown the PPI network detected among the core enrichment genes of these pathways from STRING database.

The GO terms analysis for BP highlighted 36 deregulated pathways. The top GO:BP supported the observation made in the CC analysis, as half of the terms relate to immunity responses (GO:0002377, GO:0016064), highlighting an inflammatory phenomenon occurring in the response to KD treatment. In addition, terms such as “oxidative phosphorylation, GO:0006119” and “respiratory electron transport chain, GO:0022904” are displayed in *Figure 49*.

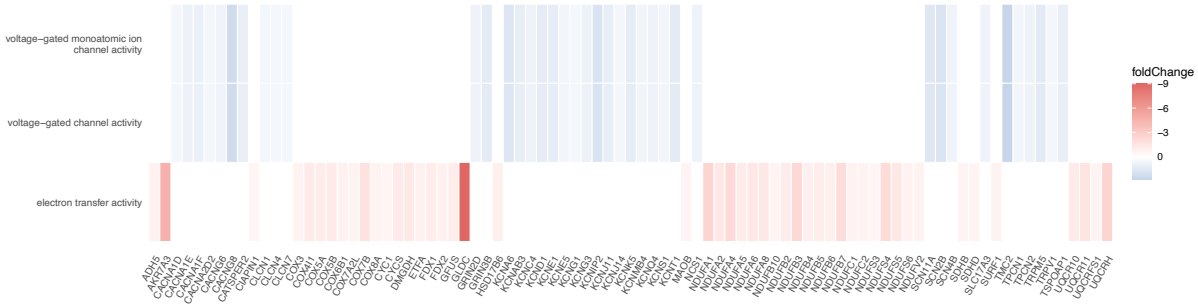
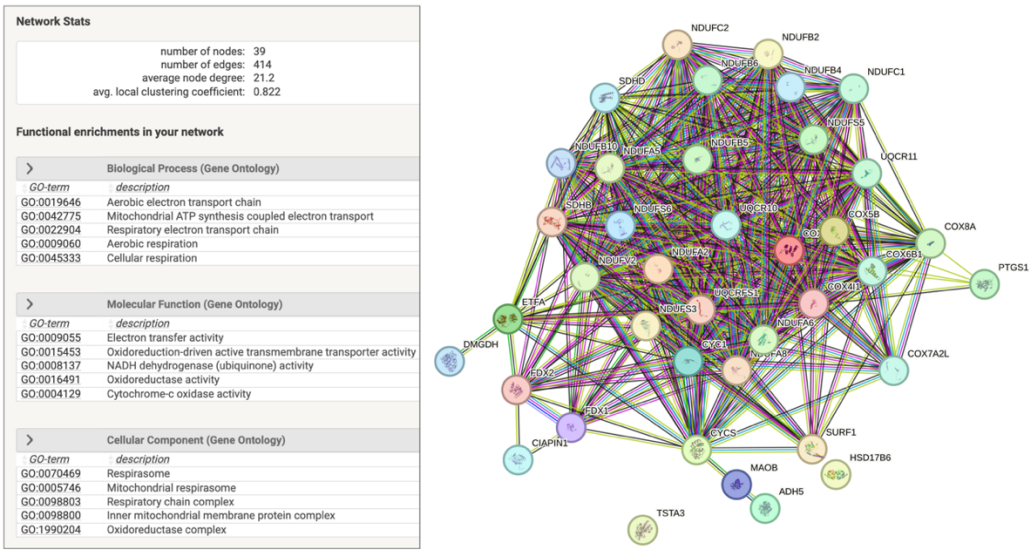


Figure 47: Heatplot for GO:MF to identify expression patterns.



Network Stats

number of nodes: 39
number of edges: 414
average node degree: 21.2
avg. local clustering coefficient: 0.822

Functional enrichments in your network

Biological Process (Gene Ontology)

GO-term	description
GO:0019646	Aerobic electron transport chain
GO:0042775	Mitochondrial ATP synthesis coupled electron transport
GO:0022904	Respiratory electron transport chain
GO:0009060	Aerobic respiration
GO:0045333	Cellular respiration

Molecular Function (Gene Ontology)

GO-term	description
GO:0009055	Electron transfer activity
GO:0015453	Oxidoreduction-driven active transmembrane transporter activity
GO:0008137	NADH dehydrogenase (ubiquinone) activity
GO:0016491	Oxidoreductase activity
GO:0004129	Cytochrome-c oxidase activity

Cellular Component (Gene Ontology)

GO-term	description
GO:0070469	Respirasome
GO:0005746	Mitochondrial respirasome
GO:0098803	Respiratory chain complex
GO:0098800	Inner mitochondrial membrane protein complex
GO:1990204	Oxidoreductase complex

Figure 48: STRING PPI network for electron transfer activity (GO:0009055) term highlighted functional enrichments such as mitochondrial respirasome and ATP synthesis.

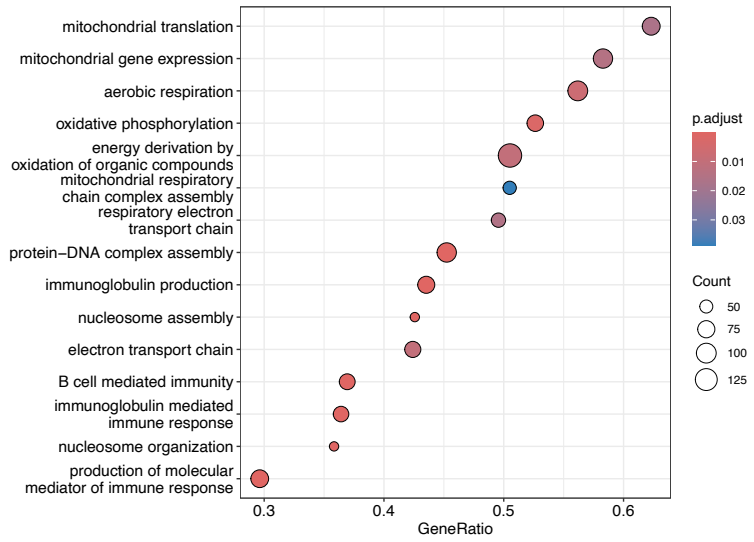


Figure 49: Dotplot of BP analysis for DEGs in good versus bad outcomes. The y-axis represents the name of the pathway, the x-axis represents the Gene Ratio, dot size represents the number of counts, and the colour indicates the adjusted p-adjust.

4.7.3 Pathway analyses: KEGG, WikiPathways and Reactome

KEGG, WikiPathways and Reactome analyses were also carried out using GSEA to evaluate global gene expression changes into biological pathways. All analyses highlighted oxidative phosphorylation and electron transport chain OXPHOS system in mitochondria among the significantly deregulated processes.

The deregulated pathways ranked for their significance are reported for Reactome database in the Ridgeplot in *Figure 50*. Besides mitochondria processes, a strong epigenetic component is represented, with DNA methylation, histone modification and various RNA-mediated processes, many more found deregulated. Moreover, mRNA Splicing (hsa03040) and mRNA processing pathways via Spliceosome were deregulated via KEGG analysis, suggesting an involvement of RNA splicing process in diet's response.

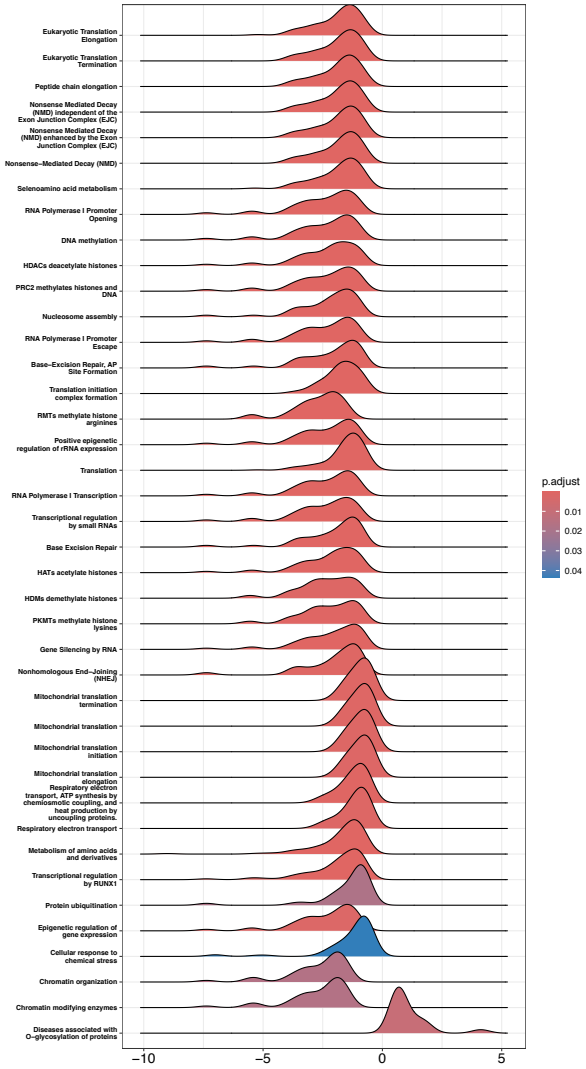


Figure 50: Ridgeplot for GSEA Reactome analysis. The y-axis represents the name of the pathway, the x-axis represents the log2FC, the shape describes the density/number of genes with a specific log2FC value, and the color indicates the adjusted p-adjust as listed in the color bar.

4.7.4 Computational and functional predictions of snoRNAs, snRNAs and lncRNAs

The detected 4 snoRNAs (SNORD3D, SCARNA5, SNORD17 and SNORD3A) were downregulated in KD responders and involved in tRNA processing (GO:0006396) mechanism, such as RNA modifications and alternative splicing (*Table 21*).
Likewise, 4 snRNAs (RNVU1-7, RNU1-1, RNU1-3 and RNVU1-18), implicated in mRNA 5'-splice site recognition, were down-regulated.
Figure 51 shows the functional enrichment pathways from STRING database for GO:CC.

Transcript_ID	gene_name	log2FC	padj
ENSG00000277947	SNORD3D	-11,13843	0,00279185
ENSG00000252010	SCARNA5	-4,44441	0,07055958
ENSG00000212232	SNORD17	-4,22419	0,07709693
ENSG00000263934	SNORD3A	-3,72617	0,08711204
ENSG00000206585	RNVU1-7	-23,446533	0,03960769
ENSG00000206652	RNU1-1	-8,8706872	0,07148720
ENSG00000207513	RNU1-3	-8,8706872	0,07148720
ENSG00000206737	RNVU1-18	-8,8706872	0,07148720

Table 19: List of snoRNAs and snRNAs in responders versus non-responders to KD.

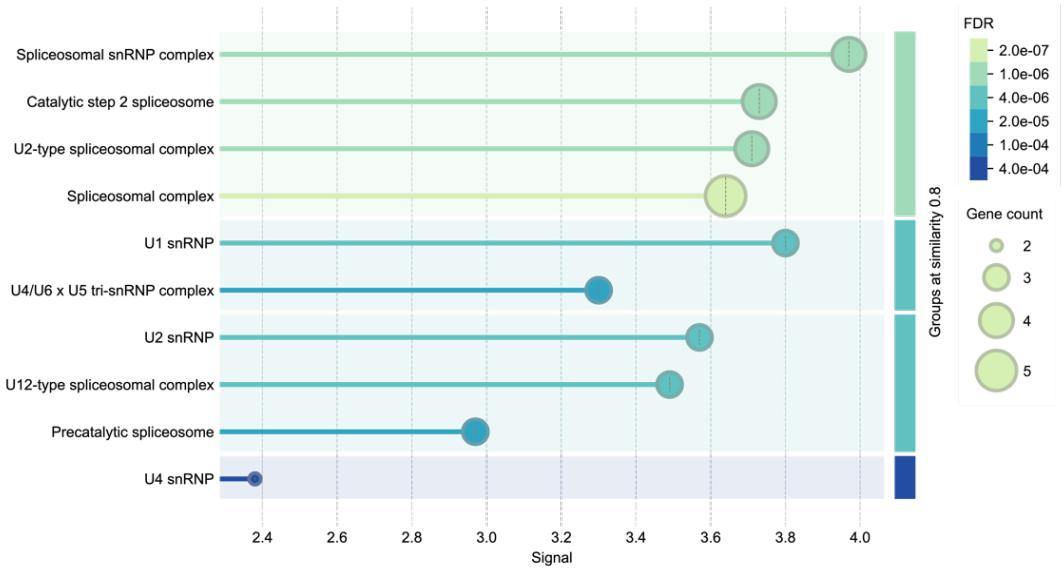


Figure 51: Functional enrichment pathways for GO:CC. The y-axis represents the name of the pathway, the x-axis represents the signal (defined as a mean between the observed ratio and -log2(FDR)), dot size represents the number of gene count, and the color indicates the False Discovery Rate (FDR), which describes how significant the enrichment is.

When focusing on lncRNAs, the comparison revealed two upregulated lncRNAs (ENSG00000228334 and ENSG00000278932) and two downregulated ones (ENSG00000273295 and ENSG00000259001), reported in *Table 22*. Notably, ENSG00000228334 and ENSG00000273295 are also detected in adults versus children comparison.

The lncRNA-gene co-expression analysis was also performed using the lncHub2, providing predictions on biological functions and secondary structures of detected lncRNA.

Focusing on BP, of particular interest is their involvement in biological pathways detected in coding transcriptome analyses, e.g. the regulation of interferon-beta production, the regulation of cytokine production involved in immune response, the regulation of sodium ion transmembrane transporter activity, and the mitochondrial respiratory chain complex assembly. KEGG analysis revealed the oxidative phosphorylation, the calcium signaling, and the spliceosome as predicted biological pathways.

In *Figure 52* are reported secondary structure of lncRNAs predicted with RNAfold.

Transcript_ID	**gene_name**	**log2FC**	**padj**
ENSG00000273295	ENSG00000273295	-10,4897	0,004411
ENSG00000259001	ENSG00000259001	-3,42429	0,050399
ENSG00000278932	ENSG00000278932	3,894561	0,043847
ENSG00000228334	ENSG00000228334	9,613834	0,000521

Table 20: List of lncRNAs in good versus bad clinical response. Log2FC and padj are displayed for each transcript.

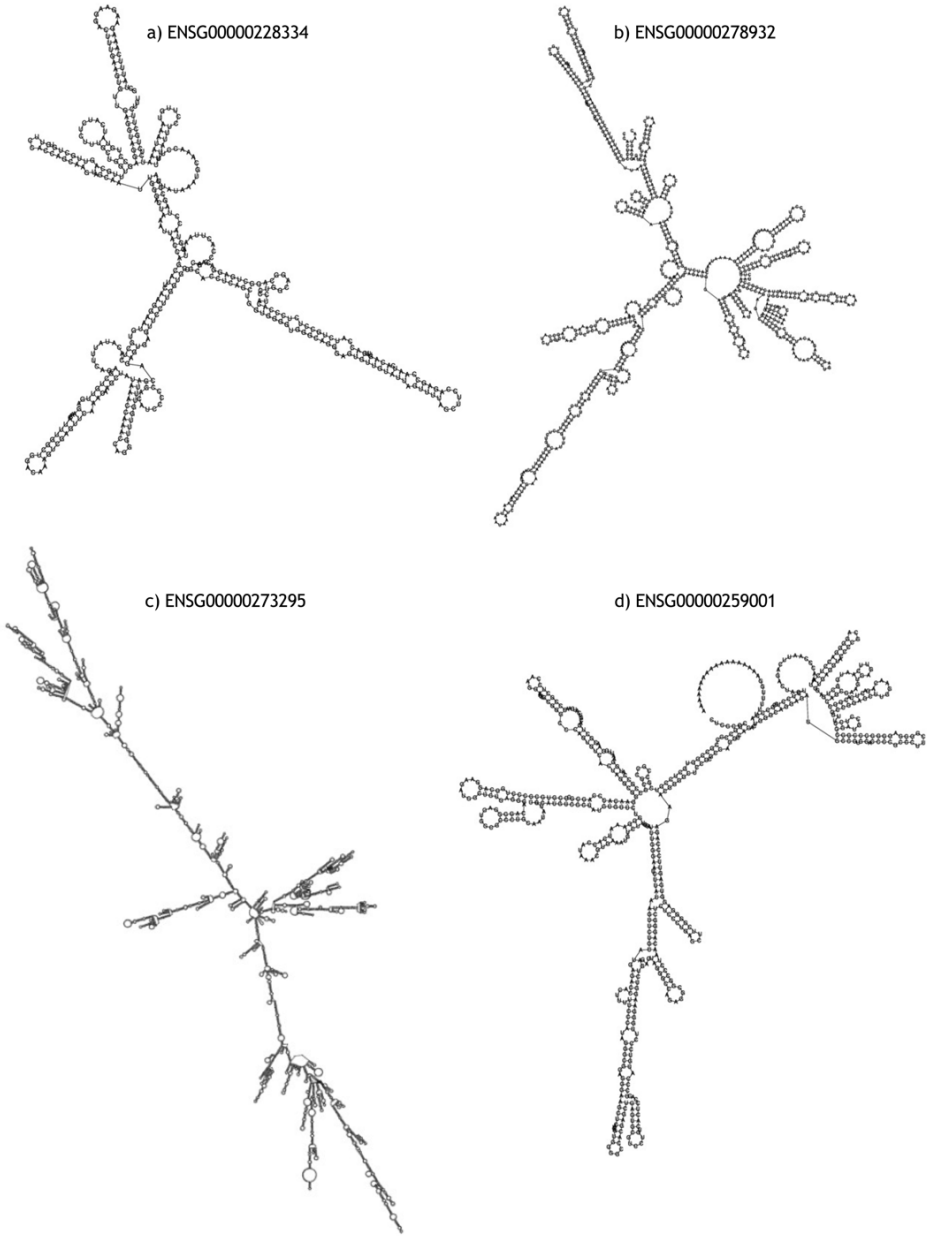


Figure 52: Predicted secondary structure of lncRNAs as obtained RNA fold web server.

4.7.4.1 Comparison between lncRNA-correlated genes and DEGs

The top 25 genes positively and negatively correlated with the identified lncRNA are compared with 68 DEGs detected in responders versus non-responders comparison. This highlighted some deregulated genes associated with lncRNA ENSG00000259001, which showed a positive correlation with two ribonucleoproteins, RN7SL1 and RPPH1, and one snoRNA (SNORD3D). RN7SL1 and RPPH1 genes, resulting downregulated in good response, are involved, respectively, in sequence receptor complex recognition (GO:0005786) and tRNA processing (GO:0008033). SNORD3A, as discussed previously,

is involved in RNA processing (GO:0006396). Interestingly, ENSG00000259001 seems to be correlated with these genes and involved in spliceosoma process.

4.8 Interferon signatures in GLUT1-DS patients

Transcriptome analyses for all three comparisons highlighted interferon signalling among the significantly deregulated processes. These gene expression data were also confirmed by type I IFN qPCR assay, BioMole. Pediatric patients with GLUT1 deficiency syndrome before the KD had higher levels of IFN signature score compared with GLUT1-DS children in diet, as shown in *Figure 53a*. Affected adults in *Figure 53b* had lower expression of IFN score compared with children untreated, while they had no significative difference related to patients underwent KD, represented in *Figure 53c*. In the comparison responders versus non-responders in *Figure 53*d, IFN scores had higher expressions in pediatric patients with bad clinical response to KD. This latter condition resulted in statistical significative difference compared with children in pre-KD, as shown in *Figure 53e*.

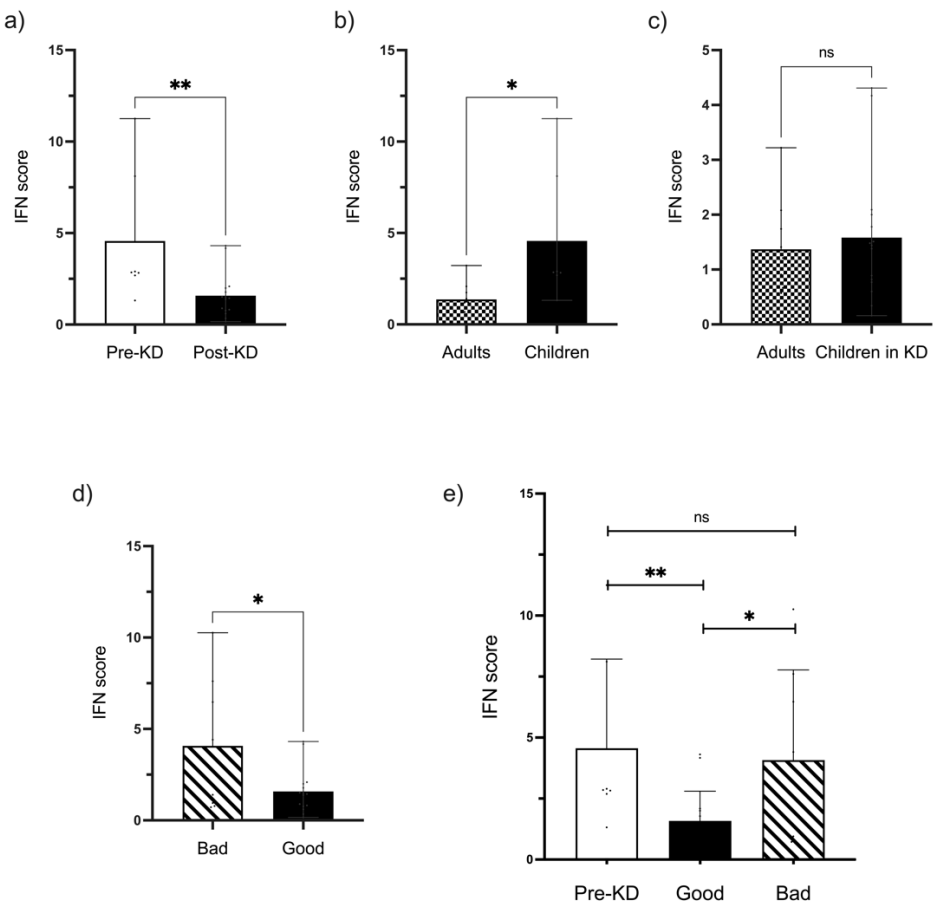


*Figure 53: Interferon (IFN)-1 signatures in GLUT1-DS patients. a) IFN scores in GLUT1-DS children pre- and post-KD; b and c) IFN scores in adults and children in pre- and post-KD, respectively; d) IFN scores in patients underwent KD with good clinical outcomes and bad ones; e) IFN scores in pre-KD, good clinical outcomes and bad ones. The IFN score of each individual is represented as single dot. Unpaired t test was performed using Prism 10 (*p<0.05; **p<0.001).*

4.9 Methylome data of GLUT1-DS children in pre- versus post-KD

In this section we investigated the DNA methylation changes occurring between GLUT1-DS pediatric patients before and post-KD treatment in order to define the molecular pathways that may directly contribute to beneficial effects of the KD.
In this experimental condition analyzed, a whole genome sequencing by ONT, along with base modification detection (including 5mC and 5mhC) and DMR analysis, was performed.

4.9.1 DNA modification profiling

As shown in *Figure 54a*, we determined the global levels of various DNA base modifications (5mC + 5hmC) in pre- (74.5%) and post-KD (75.2%). Specifically, in pre-KD condition DNA 5mC resulted 70.6% and 5hmC was 3.9%, whereas in children underwent diet, 5mC was 71.5% and 5hmC of 3.7%. Although DNA modifications levels in both conditions were very similar, their profiles distribution showed differences, as illustrated in density plots (*Figure 54b-c*).

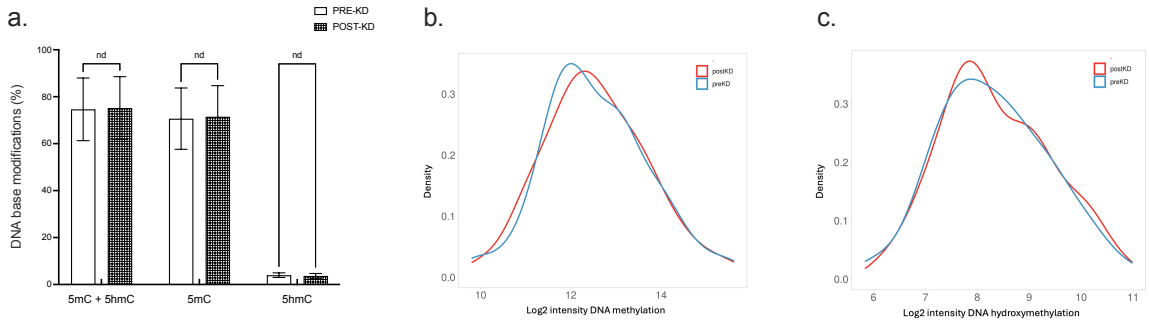


Figure 54: a) DNA modifications levels in pre- versus post-KD; b) DNA Methylation distribution: density plot of transformed data (log2) describes the methylation values of samples grouped by condition; c) DNA hydroxymethylation distribution: density plot of transformed data describes the hydroxymethylation values of pre- and post-KD conditions.

4.9.2 Differential 5mC and 5hmC DNA analysis

Ion channels data resulting from transcriptome data were used to perform differential methylation analysis by ONT adaptive sampling approach. We compared both 5mC and 5hmC differences in GLUT1-DS pediatric patient in pre- versus post-KD and classified 69 target genes in *Table 23* into different ranges based on log2FC.
MA plot, in *Figure 55a*, showed the differences in 5mC among the conditions, 59 out of 69 genes (85.5%) were hypo-methylated in pre-KD group, suggesting low methylation levels in GLUT1-DS pediatric patients before KD. Otherwise, 5hmC differences proposed a lower hydroxymethylation in children before KD compared to post-KD with 45 hypo-

hydroxymethylated (65.2%) and 24 hyper-hydroxymethylated genes (34.7%) in pre-KD condition, as displayed in *Figure 55b.*

Chr	Start location	End location	Gene	Chr	Start location	End location	Gene
chr1	160027467	160080160	KCNJ10	chr6	39178971	39239475	KCNK5
chr9	101559352	101748647	GRIN3A	chr17	7911859	7939856	KCNAB3
chr6	35933516	36034641	SLC26A8	chr15	78555520	78605269	CHRNA5
chr1	181307699	181818084	CACNA1E	chr8	27300506	27469391	PTK2B
chr3	38538062	38659687	SCN5A	chr1	40773787	40830452	KCNQ4
chr19	54150108	54183160	TMC4	chr14	92312581	92511481	SLC24A4
chr22	36243133	36277525	APOL1	chr14	77171798	77269495	TMEM63C
chr10	103443240	103468900	CALHM1	chr17	39953004	39987768	GSDMA
chr21	38219926	38317357	KCNJ15	chr12	121122876	121198032	P2RX7
chr21	34436688	34522210	KCNE1	chr19	13196442	13516479	CACNA1A
chr10	76859602	77647808	KCNMA1	chr2	166185185	166385987	SCN9A
chr15	79950892	79981196	BCL2A1	chr8	143543387	143573062	GSDMD
chr11	6224793	6254976	CNGA4	chr12	49529030	49568337	KCNH3
chr21	34658994	34728223	CLIC6	chr9	74712495	74897921	TRPM6
chr17	70159532	70190044	KCNJ2	chr15	34219784	34348057	SLC12A6
chrX	9776429	9959443	SHROOM2	chr11	69038932	69100597	TPCN2
chr11	113888923	113959079	HTR3B	chr7	102423575	102466825	ORAI2
chrX	109613700	109635172	KCNE5	chr14	73126417	73233691	PSEN1
chr11	118123377	118162823	SCN4B	chr10	101815974	101853800	KCNIP2
chrX	48951380	48981844	KCND1	chr6	39289001	39324419	KCNK17
chr6	116451375	116473771	CALHM6	chr22	20999799	21038008	P2RX6
chr17	3886592	3926465	P2RX1	chr12	51581233	51822864	SCN8A
chr9	33373191	33412568	AQP7	chr11	22182473	22293357	ANO5
chr15	58128169	58195911	AQP9	chr21	44343621	44452644	TRPM2
chr11	61939821	61975515	BEST1	chr15	30350566	30403900	CHRFAM7A
chr1	111760662	111999668	KCND3	chr20	34992404	35102807	TRPC4AP
chr2	165229414	165402304	SCN2A	chr19	579881	627159	HCN2
chr7	151038292	151062756	ASIC3	chr7	2621986	2674802	TTYH3
chrX	10146975	10247660	CLCN4	chr7	75976831	76004595	TMEM120A
chrX	37770059	37823461	CYBB	chr20	45081214	45111127	KCNS1
chr12	109773087	109843398	TRPV4	chr3	43355848	43701594	ANO10
chr11	74444841	74477549	KCNE3	chr1	161215939	161240746	TOMM40L
chr6	33562552	33590276	BAK1	chr6	31720581	31747318	CLIC1
chr2	64978479	65033865	SLC1A4	chr22	50160731	50190295	PANX2
chr1	150564558	150589610	MCL1				

Table 21: BED file containing genomic regions of target genes used for ONT adaptive sampling approach.

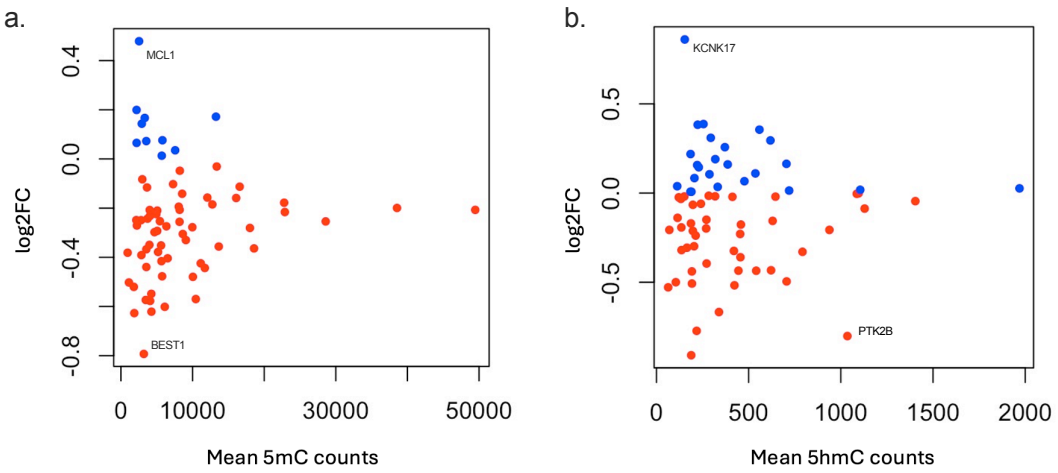


Figure 55: MA plots to visualize differential methylation (a) and hydroxymethylation (b) data in pre- versus post-KD. Genes are indicated through a color code, where in blue are represented hyper-methylated or hyper-hydroxymethylated genes and in red the hypo-methylated or hypo-hydroxymethylated ones.

Specifically, the genes that exhibited the most significant variations in DNA methylation status (6 hyper-methylated and 6 hypo-methylated) are reported in *Table 24*. Notably, *MCL1* showed a methylation of 60.93% in pre-KD condition, whereas a difference of -10.7% was displayed in post-KD group. Also, *KCNJ2*, *CLIC6*, *KCNS1, TMC4*, and *SCN4B* genes had a similar trend, highlighting hyper-methylation in pre-KD versus post-KD comparison. On the other hand, *BEST1*, *SLC1A4*, *P2RX1*, *CHRNA5*, *KCNQ4* and *GRIN3A* genes exhibited methylation differences within range -8.97% - 5.69% indicating hypo-methylation in pre-KD condition. Other genes such as *CLCN4* (0.32%), *SLC24A4* (0.21%), *HTR3B* (0.1%), *CACNA1E* (0.08%), *TRPV4* (-0.61%) and *PANX2* (-0.16%) displayed less marked differences.

Gene_name	**5-methylcytosine (%)**	
	pre-KD	**post-KD**
MCL1	60,93	50,23
KCNJ2	65,89	56,3
CLIC6	74,84	65,85
KCNS1	54,63	49,29
TMC4	72,77	66,07
SCN4B	55,51	50,8
BEST1	46,09	55,06
SLC1A4	53,79	63,89
P2RX1	59,34	69,67
CHRNA5	77,01	84,07

KCNQ4	57,74	62,77
GRIN3A	74,56	80,25

Table 22: List of genes that exhibited the most significant variations in 5mC.

4.9.3 Integration of gene expression and DNA methylation datasets

To investigate functional genes and how DNA methylation levels impact on gene expression, we performed a comparison based on data integration. By intersecting with the set of low methylation levels and upregulated expression genes, the *GRIN3A* gene is unveiled in pre-KD condition. Indeed, we found the glutamate ionotropic receptor NMDA-type subunit 3A (OMIM #606650; 9q31.1), which is related to glutamatergic system, among the most hypo-methylated and upregulated gene in GLUT1-DS children before treatment compared to patients underwent KD. Methylation status of *GRIN3A* promoter 1 (chr9:101738637-101738696) for 23G3 (pre-KD) and 23G55 (post-KD) samples is displayed on IGV in *Figure 56*.

In literature, the *GRIN3A* gene is involved in controlling synaptic activity and plasticity. Its excessive activity may result in increased neuronal excitability, contributing to developmental delay and susceptibility to seizures. Moreover, gain-of-function or loss-of-function mutations in *GRIN3A* gene have been associated with a spectrum of epileptic disorders, including early-onset epilepsy and generalized epilepsy (Santos-Gómez et al., 2021; Strehlow et al., 2019).

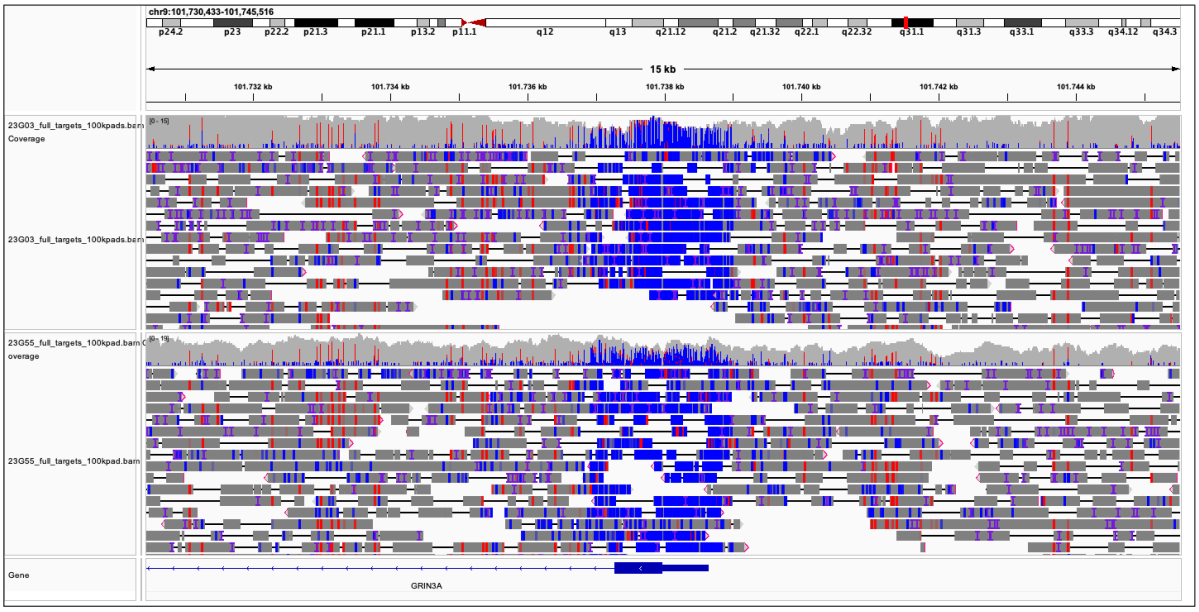


Figure 56: Viewing methylation status in IGV of GRIN3A promoter 1 in position chr9:101738637-101738696 for samples in pre-KD (23G3) and post-KD (23G55). In blue are displayed unmethylated CpG and in red methylated CG.

5. Discussion

GLUT1-DS is a rare, effectively treatable, and not well-known neurometabolic disease. It is due to heterozygous loss-of function mutations in the *SLC2A1* gene, encoding the GLUT1 protein, which supplies glucose to the brain. A genetic defect in this transporter causes cerebral energy deficiency that results in a complex spectrum of neurological symptoms, including seizures, movement disorders, and cognitive developmental delays. Although many clinical and biochemical features have been investigated and unraveled since its first description by De Vivo in 1991 (De Vivo et al., 1991), there are still some aspects, including (a) clinical variability and wide spectrum of *SLC2A1* variations, (b) phenotype age-dependent changes, and (c) response to KD treatment, which remain unclear and/or not totally understood.

In the light of the above, the first aim of this proposal was to explore molecular and biochemical data of 27 pediatric patients clinically affected by GLUT1-DS from both sporadic and familial cases. Indeed, the detection and downstream variant interpretation analysis have allowed the identification of disease-related mutations (10 out of a total of 25 identified variants) never been reported before, broadening the genotypic heterogeneity found in the *SLC2A1* gene and suggesting their potential implications on GLUT1 stability, and binding and transport of glucose. Notably, the novel *de novo* splice site variant c.114+1G>A identified in patient 3 affected donor splice site of exon 2, thus leading to exons 1-3 junction, exon 2 skipping, with formation of an aberrant GLUT1 transcript.
Moreover, the mapping of 25 different variants detected in our cohort to GLUT1 protein structure highlighted mutational hotspot (Pascual et al., 2008) that involves TMH4 and TMH5 domains encoded by exon 4 of the *SLC2A1* gene, as previously reported by Pascual *et al.* (2008). These findings, supported by protein function analysis and both biochemical and clinical data (patients 8, 9 and 10), suggested that structural alterations in this vulnerable region of the GLUT1 protein could affect the glucose uptake into the brain.
The broad phenotypic variability and molecular heterogeneity observed in our study population enabled us to delineate strong correlations between specific mutations in *SLC2A1*, biochemical findings and clinical outcomes. Splice site and frameshift variants, resulting in 50% loss of GLUT1 activity, are related to a more severe phenotype characterized by early-onset classical phenotype and low CSF/blood glucose ratios (below 0.37 mg/dL).

The investigation of the transcriptional differences reported in *SLC2A1*-mutated adults and infants, before and after KD treatment, was another purpose of this research work. Indeed, the identification of a subset of DEGs, as well as gene isoform switching, in adults and

children, both untreated, allowed to explore specific molecular pathways responsible for the variable clinical course of disease, including why symptoms change over time, from infancy to adulthood, and what happens to aged patients. Moreover, the comprehensive analysis of the transcriptional differences, as well as DNA methylation status, between pediatric patients before the treatment and those underwent KD, specifically, highlighted DE RNAs related to diet in GLUT1-DS patients. These molecular targets allowed to define how ketosis state improves most clinical symptoms and why in some patients KD fails, rendering GLUT1 deficiency not always a treatable disease, and even could become useful for the development of both predictive biomarkers and therapies based on precision medicine.
Specifically, RNA-Seq was performed on PBMC's obtained from untreated adults (n=6), untreated infants (n=3) and infants underwent KD (n=12) with GLUT1 deficiency. Three experimental conditions were subsequently analyzed: (a) the differences occurring between adults and pediatric patients both untreated, (b) the differences occurring between pediatric patients in pre- and under-KD, and (c) the differences occurring between children in KD with good clinical outcomes and bad responses. For each comparison, a global bioinformatics analysis of DEGs and pathways in which they are involved was carried out.

In adults versus children affected by GLUT1-DS, the findings suggest that the age profoundly influences the whole gene expression in patients, and these transcriptional changes age-related could be responsible for the variable clinical course of GLUT1 deficiency observed in aged patients.
69 deregulated genes were identified, and of these 10 were pseudogenes, 29 were non-coding RNAs, whilst 30 were protein-coding genes. It is extremely interesting to note that most of the top deregulated genes consists in ncRNAs, supporting that this class is of critical significance in aging and could thus be of great relevance in clinical course of GLUT1 disease (Kour & Rath, 2016; Sherazi et al., 2023). Amongst the 13 detected lncRNAs, RMRP was defined as T cell-related lncRNA in epilepsy (Sharifi et al., 2022), whilst PAX8-AS1 was related to oxidative phosphorylation and calcium signaling, processes resulting from enrichment analyses, discussed below. This lncRNA was also found to be differentially expressed in pre- versus post-KD comparison, suggesting a specific role in onset and disease progression, warranting further functional studies.
GO terms enrichment in MF, CC and BP revealed a significant implication for immune-related functions with a downregulation in adults, potentially reflecting a mitigated inflammatory response age-related. Other relevant pathways were implicated in mitochondrial function, particularly oxidative phosphorylation and electron transport chain processes. Reduced inflammatory responses and stabilized metabolic demands may correlate with the changes in clinical symptoms reported in adults, like a possible reduction

in seizure frequency (Giugno et al., 2024). It is relevant to emphasize in this context that this observed shift may be attributed to several interconnected biological and developmental factors. The brain's energy demands differ significantly between infants and adults. In infants, glucose is a primary energy source critical for rapid brain development. Adults, however, have a more stable neural architecture and may rely on alternative pathways more effectively (e.g. lipid metabolism) as compensatory mechanisms given also by long-term stress exposure.

A subsequent analysis, through Reactome and WikiPathways, showed that a significant number of deregulated pathways correlated with different types of epigenetic changes (e.g. DNA methylation, histone acetylation and deacetylation, gene silencing by RNA). This suggests that these specific altered molecular signatures could be implicated in the progression of GLUT1-DS and its clinical variability between adults and children. Specifically, these changes may influence the expression of key genes, as observed, without altering their DNA sequences, reflecting a layer of regulation sensitive to environmental.

Moreover, KEGG highlighted the RNA splicing processes via spliceosome, showing many downregulated snoRNAs involved in RNA modifications and alternative splicing, and underscoring a broader dysfunction in the post-transcriptional machinery. A deeper insight into these dynamics was performed on alternative splicing and isoform switching. Adults versus children exhibited significant isoform usage differences across 62 genes, many affecting protein domains and coding potential, and suggesting post-transcriptional regulation. Interestingly, *SUOX* and *NACC1* genes, both associated with epilepsy, demonstrate isoform-specific alterations, which could impact protein function and seizure susceptibility (Sass et al., 2010; Schoch et al., 2017); *HLA-DQA1*, linked to immune pathways (Horta et al., 2015), shows a shift in isoform usage, potentially affecting immune regulation in GLUT1-DS. Notably, these findings suggest that post-transcriptional regulation, rather than changes in overall gene expression, contributes significantly to shape the clinical features and progression of GLUT1-DS. However, it’s important to note that further analyses are required to fully define which genes/pathways are related to aging and which to GLUT1 disease.

By comparing GLUT1-DS pediatric patients in pre- versus post-KD conditions, the analyses highlighted significant transcriptional and isoform changes, and epigenetic modifications as core mechanisms modulated by KD in GLUT1 deficiency.

PCA and heatmap analyses from whole transcriptome data showed clear clustering and separation between pre- and post- samples, supporting the hypothesis that KD induces broad gene expression changes. Most DEGs (50 upregulated and 19 downregulated) were linked to ion channel signaling and immunity pathways. GO analysis for CC, indeed, displayed a

significant implication for immune response with an upregulation in pre-KD group, with elevated expression of interferon-signaling genes (e.g. *IFIT1*, *IFIH1*, *RSAD2*). As confirmed by IFN qPCR assay, a reduction of these immune-related transcripts in post-KD condition may reflect a normalization of inflammatory states, potentially supporting the clinical symptoms improvement observed in patients underwent KD. This finding highlight that the type I IFN signature may contribute to pathogenesis of GLUT1-DS and could potentially serve as a diagnostic score for this disease. However, it's important to note that further research is needed to fully understand the role of IFN in GLUT1 disease.

Moreover, lncRNA-gene co-expression analysis, performed on 8 lncRNAs using lncHub2, identified interactions with immune genes, such as LINC02555 correlating with cytokine and interferon-signaling genes (*RSAD2, IFIT3, HERC5* and *SAMD9L*), as well as pathways related to ketone biosynthesis. Although studies linking lncRNAs to ketone biosynthesis are limited, research highlighted their role as key regulators of metabolic pathways, including lipid metabolism and energy homeostasis (Gluba-Sagr et al., 2024; T.-N. Zhang et al., 2020). For example, lncRNAs can interact with transcription factors or chromatin modifiers to regulate genes encoding enzymes involved in fatty acid oxidation, a process intrinsically linked to ketogenesis. Considering these data, which suggest a potential indirect influence on transcriptional networks governing ketogenesis, targeted experiments are needed to elucidate their specific molecular interactions and therapeutic potential in disorders like GLUT1-DS.

A subsequent analysis, through Reactome and WikiPathways, emerged amongst the deregulated processes crucial epigenetic mechanisms such as DNA methylation, histone modifications and RNA post-transcriptional modifications. Pre-KD patients, indeed, showed by KEGG analysis upregulated mRNA splicing processes, highlighting isoform switching as a potential contributor to symptom improvement. In accordance with these findings, alternative splicing and switching isoform analysis revealed 325 isoforms switching in 281 genes, predominantly protein-coding. The enrichment analysis on all switching genes showed pathways related to gene expression regulation and metabolic and mitochondrial processes, whilst analysis for HPO highlighted terms like acute encephalopathy and abnormal sodium homeostasis, aligning with GLUT1 deficiency. Interestingly, an in-depth analysis revealed *P2X7*, a gene encoding an ATP-gated ion channel, involved in an isoform switch rather than differential expression. As reported in qPCR assay, the canonical coding isoform is replaced by a short non-coding isoform post-KD, potentially altering its role in energy and ion homeostasis. In literature is described that *P2RX7* gene, activated by extracellular ATP, plays a role in neuroinflammatory responses and cell signaling, modulating the release of pro-inflammatory cytokines like IL-1β (Abdullah et al., 2024; Pegoraro et al., 2024). Its activity increases under conditions of metabolic stress, such as

those seen with GLUT1 deficiency in pre-KD group. It is extremely interesting to note that both *SLC2A1* and *P2X7* genes are implicated in epilepsy: GLUT1 deficiency reduce energy supply for neurons, while P2RX7 contributes to neuronal hyperexcitability and cell death during seizures (Alves et al., 2024). Potential links between GLUT1 and P2RX7 are various: i) in conditions of GLUT1 dysfunction, reduced glucose availability leads to compromised ATP production; however, cellular stress may trigger the release of ATP into the extracellular space, activating P2X7 receptors. Excessive P2X7 activation can lead to neuroinflammation, worsening neuronal health and potentially contributing to seizure activity seen in GLUT1-DS; ii) GLUT1 deficiency may create a pro-inflammatory state due to increased oxidative stress, P2RX7 amplifies this inflammatory response by promoting cytokine release. This interaction could exacerbate brain dysfunction involving either pathway; iii) GLUT1 is crucial for maintaining BBB function; impaired glucose transport may compromise the BBB, allowing inflammatory mediators to enter the brain and activate P2X7, further promoting inflammation. The interaction between GLUT1 and P2RX7 remains a promising area for exploring the metabolic-inflammatory nexus with therapeutics implications in GLUT1 disease. For example, inhibiting P2RX7 may help reduce neuroinflammation and neuronal damage secondary to GLUT1-related metabolic dysfunction, as well as therapies combining KD and P2X7 antagonists could offer synergistic benefits in managing neuroinflammation and seizures.

In accordance with ion channel signaling deregulation detected in RNA-Seq dataset, WGS by ONT revealed altered methylation patterns in key genes such as *GRIN3A* (a gene encoding the GluN3A subunit of NMDA receptors), which was found significantly hypo-methylated and upregulated in pre-KD group. Interestingly, *GRIN3A* and *SLC2A1* share molecular pathways in epilepsy, particularly through their respective roles in pathways regulating synaptic function and energy metabolism, suggesting a potential crosstalk in epileptic pathophysiology (XiangWei et al., 2019). A dysfunction in either gene (1) disrupts the delicate balance of excitatory and inhibitory neurotransmission, which is critical in preventing seizures, (2) can impair brain metabolism and exacerbate seizure susceptibility. Moreover, both *GRIN3A* and *P2RX7* genes contribute to calcium signaling, highlighting potential mechanistic connections. NMDA receptors, indeed, regulate calcium influx critical for synaptic plasticity, and P2X7 receptors mediate calcium entry upon activation by ATP, often in response to cellular stress or damage, as discussed previously. Dysregulation of calcium signaling from either pathway can lead to neuronal hyperexcitability and seizures. Even more interesting is the potential interaction about neuroinflammation and excitatory balance. P2RX7's role in neuroinflammation may exacerbate excitotoxicity mediated by dysfunctional NMDA receptors. For instance, ATP release during seizures might hyperactivate P2X7, compounding the excitatory imbalance caused by GRIN3A

deregulation (Grassi, 2020; Zheng et al., 2024). Although these insights underline both *P2RX7* and *GRIN3A* genes as promising targets in tackling neuroinflammation-driven disorders, such as GLUT1 deficiency, targeted experiments are needed to elucidate their specific molecular interactions.

The last transcriptome data analysis of GLUT1-DS children who underwent KD treatment reveal key molecular differences between responders and non-responders to KD, which might explain why KD is effective in some cases and less so in others.
Among 68 DEGs, the majority were downregulated in responders, including 48 protein-coding genes, 16 ncRNAs, and 4 pseudogenes. GO analysis highlighted deregulated pathways in immunity and mitochondrial functions in responders versus non-responders. Indeed, the observed downregulation of immune-related genes (e.g. *IGKV* and *IGHV* genes) in patients with good clinical outcomes suggested the suppression of pathways related to immune responses, such as cytokine production and interferon-beta regulation, as shown by IFN qPCR assay. Inflammation is often linked to impaired metabolic and neurological outcomes in several conditions, including GLUT1-DS. By mitigating systemic or located inflammation, KD may improve neuronal function and stability. On the other hand, the downregulation of oxidative stress pathways and enhancement of mitochondrial efficiency (as seen in responders) suggests a metabolic shift toward optimized energy use, critical for GLUT1-DS patients, who suffer from impaired glucose transport to the brain. This dual impact of reduced inflammation and improved metabolic efficiency likely contributes to the observed benefits in GLUT1-DS responders. Moreover, Reactome enrichment revealed pathways like oxidative phosphorylation and electron transport chain among deregulated processes, consistent with KD's impact on mitochondrial energy metabolism.
A subsequent analysis, through KEGG, highlighted the spliceosome as a downregulated process, showing many downregulated protein-coding genes (e.g. *ISY1-RAB43* implicated in mRNA Splicing - Major Pathway) and ncRNAs such as snoRNAs (e.g. SNORD3A, SNORD17) and snRNAs (e.g. RNU1-1, RNU1-3) involved in RNA modifications and splicing, emphasizing the role of RNA-mediated processes in the response to KD. As well as this, the detected lncRNAs, besides being associated with key biological pathways including interferon production, correlated with genes implicated in RNA processing. Notably, the involvement of lncRNAs suggests potential regulatory roles in KD response, warranting further functional studies.

Overall, the findings presented in this study highlight the complex interplay that transcriptional variations, isoform switching and epigenetic modifications could have in the phenotype age-related changes and response to KD treatment in GLUT1-DS. The work

provides a comprehensive analysis of clinical, molecular and biochemical data of pediatric and adults patients, together with a database of which these DEGs are, what function they exert, which pathways they influence and their potential disease implications. Specifically, interferon signatures, mitochondrial signaling, and alternative splicing mechanisms emerged as novel and suggestive findings among the disease-related processes. It will be of great significance for future analyses seeking to identify new molecular targets in GLUT1-DS progression and/or response to KD, providing an essential starting point for further omics analyses, including methylome, and specific functional evaluations.

6. Conclusion

The clinical improvements observed in GLUT1-DS patients regarding seizure frequency and severity, whether in response to ketogenic diet or during adulthood, may be related to a reduction in inflammatory pathways involving type I interferons, which have an impact on mitochondrial function and signaling, as well as on neuronal excitability. Another significant molecular process contributing to mitigate clinical signs, highlighted by this research study, is the alternative splicing of key channel genes, such as P2X7 receptor which, in turn, linked to inflammasome pathways.

In conclusion, by shifting energy metabolism from glucose to ketone bodies, KD moderates glycolysis-dependent inflammatory processes and enhances ion channel activity, thereby bypassing GLUT1 and effectively compensating for the glucose deficit.

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Annex A – Publications

Rey, F., Messa, L., Berardo, C., **Mauri, A.,** Zuccotti, G., Cereda, C., & Carelli, S. (2024). Gene expression analysis in subcutaneous adipose tissue reveals a predominant influence of lncRNAs during growth. Genes & diseases, 12(2), 101351. https://doi.org/10.1016/j.gendis.2024.101351

Moresco, G., Rondinone, O., **Mauri, A.,** Gorgoglione, R., Graziani, D. M. G., Dziuback, M., Miozzo, M. R., Sirchia, S. M., Pietrogrande, L., Peron, A., & Fontana, L. (2024). A novel frameshift TBX4 variant in a family with ischio-coxo-podo-patellar syndrome and variable severity. Genes & genomics, 10.1007/s13258-024-01589-5. Advance online publication. https://doi.org/10.1007/s13258-024-01589-5

Olivotto, S., Freddi, A., Previtali, R., **Mauri, A.,** Cereda, C., De Amicis, R., Bertoli, S., Doneda, C., & Veggiotti, P. (2024). Stroke and Stroke-Like Episodes: Recurrent Manifestations in GLUT1 Deficiency Syndrome. Pediatric neurology, 157, 118–126. https://doi.org/10.1016/j.pediatrneurol.2024.05.024

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Annex B – Abstracts and oral presentations

- **Mauri A,** Messa L, Previtali R, Biganzoli D, Visceglia MC, Olivotto S, Veggiotti P, Cereda C. Impatto della dieta chetogenica sulla sindrome da deficit di GLUT1: identificazione dei meccanismi molecolari responsabili dei miglioramenti del fenotipo in pazienti pediatrici. Oral presentation. SIMMESN, October 15-18, 2024
- **Mauri A,** Pocaterra R, Vasco A, Postorivo D, Luisella A, Montrasio C, Cereda C. Selezione di un pannello di geni per lo screening neonatale genomico. SIMMESN, October 15-18, 2024
- Montrasio C, **Mauri A**, Postorivo D, Meta A, Tamburello G, Pratiffi E, Cannella L, Fazzone S, Fiumani F, Vasco A, Lucchi S, Alberti L, Cereda C. Screening Neonatale SMA in Lombardia: luci e ombre. SIMMESN, October 15-18, 2024
- Messa L, Pinoli P, Bernasconi A, Barret N, Bikbov B, Vasco A, **Mauri A**, Cappiello C, Bregonzio M, Alberti L, Carelli S, Cereda C. Integrazione di Dati Genomici, Metabolici e Intelligenza Artificiale nello Screening Neonatale: Il Progetto BETTER per Migliorare Diagnosi e Trattamenti delle Malattie Metaboliche Rare. SIMMESN, October 15-18, 2024
- **Mauri A,** Messa L, Previtali R, Biganzoli D, Berardo C, Olivotto S, Visceglia MC, Meta A, Vasco A, Carelli S, Veggiotti P, Cereda C. Ketogenic diet therapies for drug-resistant epilepsy might affect ion channels activity through the combination of both epigenetic changes and splicing events. Oral presentation. ECHG, European Congress on Human Genetics, London, UK, October 6-7, 2024. ***Best communication award.***
- **Mauri A,** Messa L, Previtali R, Biganzoli D, Berardo C, Olivotto S, Visceglia MC, Meta A, Vasco A, Carelli S, Veggiotti P, Cereda C. Ketogenic diet treatment in GLUT1-DS patients: identification of ion channel signaling deregulation related to both epigenetic

changes and splicing events. Oral presentation. Brayn, Brainstorming research assembly for young neuroscientists. Verona, Italy, October 9-10, 2024

- Esposito L, Rey F, **Mauri A**, Allevi R, Mazzucchelli S, Zuccotti G, Vaia Y, Bertini E, Nicita F, Tonduti D, Rosati J, Cereda C, Carelli S. New transcriptional and functional insights in RNA polymerase III-related Leukodystrophy-affected patients. Brayn, Brainstorming research assembly for young neuroscientists. Verona, Italy, October 9-10, 2024
- Maghraby E, Rey F, Esposito L, Leone M, **Mauri A,** Orcesi S, Allevi R, Mazzucchelli S, Zuccotti G, Tonduti D, Carelli S, Cereda C. Novel frontiers in Aicardi-Goutières syndrome: association between a RNU7-1 variant and histone dysfunctions. Brayn, Brainstorming research assembly for young neuroscientists. Verona, Italy, October 9-10, 2024
- Berardo C, Dardi M, **Mauri A**, Messa L, Rey F, Zuccotti GV, Carelli S, Cereda C. Epigenetic implication in the differentiation of human pediatric adipocytes. SIGU, Italian Society of Human Genetics. Padova, Italy, October 2-4, 2024
- **Mauri A,** Messa L, Previtali R, Biganzoli D, Visceglia MC, Olivotto S, Veggiotti P, Cereda C. Ketogenic diet treatments in GLUT1-DS patients: identification of ion channel signaling deregulation related to both epigenetic changes and splicing events. SIGU, Italian Society of Human Genetics. Padova, Italy, October 2-4, 2024
- Messa L, Pinoli P, Bernasconi A, Barret N, Bikbov B, Vasco A, **Mauri A**, Cappiello C, Bregonzio M, Alberti L, Carelli S, Cereda C. The BETTER project for the advancement of clinical and genetic data: impact on newborn screening. SIGU, Italian Society of Human Genetics. Padova, Italy, October 2-4, 2024
- Fiumani F, **Mauri A**, Pocaterra R, Visceglia MC, Montrasio C, Cereda C. Long-read sequencing from dried blood spots: a useful application in Newborn Screening. SIGU, Italian Society of Human Genetics. Padova, Italy, October 2-4, 2024
- Maghraby E, Rey F, Esposito L, Leone M, **Mauri A**, Orcesi S, Allevi R, Mazzucchelli S, Zuccotti G, Tonduti D, Carelli S, Cereda C. Novel players in AGS7 and AGS9 pathogenic variants in Aicardi-Goutières Syndrome. SIGU, Italian Society of Human Genetics. Padova, Italy, October 2-4, 2024
- Pocaterra R, **Mauri A**, Vasco A, Postorivo D, Alberti L, Montrasio C, Cereda C. Genomic Newborn Screening: a consensus gene panel emerging from literature review. SIGU, Italian Society of Human Genetics. Padova, Italy, October 2-4, 2024
- Montrasio C, **Mauri A**, Postorivo D, Meta A, Tamburello G, Pratiffi E, Cannella L, Fazzone S, Fiumani F, Vasco A, Lucchi S, Alberti L, Cereda C. Screening Neonatale SMA in Lombardia: i primi risultati dello studio pilota. SIGU, Italian Society of Human Genetics. Padova, Italy, October 2-4, 2024
- Montrasio C, **Mauri A**, Vasco A, Alberti L, Lucchi S, Fazzone S, Tamburello G, Carelli S, Tagliaferri F, Furlan F, Menni F, Pretese R, Crescitelli V, Gasperini S, Rovelli V, Zuccotti G, Saielli L, Verduci E, Cereda C. Correlazione genotipo-fenotipo biochimico

di deficit di beta-chetotiolasi. SIGU, Italian Society of Human Genetics. Padova, Italy, October 2-4, 2024

- **Mauri A,** Messa L, Previtali R, Biganzoli D, Berardo C, Olivotto S, Visceglia MC, Meta A, Vasco A, Carelli S, Veggiotti P, Cereda C. Ketogenic diet therapies for drug-resistant epilepsy might affect ion channels through the combination of both epigenetic changes and splicing events. ESHG, European Society of Human Genetics, Berlin, Germany, June 1-4, 2024
- Berardo C, Vasco A, Lucchi S, Cappelletti L, Saielli L, Pratiffi E, Fazzone S, Meta A, Benedetti E, **Mauri A**, Carelli S, Furlan F, Rovelli V, Menni F, Gasperini S, Alberti L, Cereda C. Investigating the relationship between genotype and biochemical profiles in inborn errors of metabolism. ESHG, European Society of Human Genetics, Berlin, Germany, June 1-4, 2024
- Berardo C, **Mauri A,** Messa L, Rey F, Zuccotti G, Cereda C, Carelli S. Role of lncRNAs in the differentiation of human pediatric adipocytes through Wnt pathway. Obes Facts 2024;17(suppl 1):7–515 DOI: 10.1159/000538577. 31th European Congress on Obesity (ECO 2024), May 12-15, 2024
- **Mauri A,** Messa L, Previtali R, Biganzoli D, Berardo C, Olivotto S, Visceglia MC, Meta A, Vasco A, Carelli S, Veggiotti P, Cereda C. Ketogenic diet therapies for drug-resistant epilepsy might affect ion channels activity through the combination of both epigenetic changes and splicing events. Oral presentation. HGM, Human Genome Meeting, Rome, Italy, April 8-10, 2024
- **Mauri A,** Alberti L, Berardo C, Esposito L, Biganzoli D, Iascone M, Lucchi S, Benedetti E, Carelli S, Pretese R, Gasperini S, Cereda C. Identificazione di una nuova variante di splicing nel gene HADHB causativa del deficit di proteina trifunzionale mitocondriale. SIMMESN, November 15-17, 2023
- **Mauri A,** Berardo C, Meta A, Carelli S, Alberti L, Cereda C. Towards genomic-NBS: technical feasibility of NGS starting from dried blood spots. APHL/ISNS Newborn Screening Symposium Sacramento, California, Hybrid, October 15-19, 2023
- Berardo C, **Mauri A,** Lucchi S, Cappelletti L, Pratiffi E, Fazzone S, Meta A, Benedetti E, Saielli L, Alberti L, Cereda C. Expanded newborn screening: a 5-year experience eof lombardia region (Italy). APHL/ISNS Newborn Screening Symposium Sacramento, California, Hybrid, October 15-19, 2023
- **Mauri A,** Berardo C, Meta A, Biganzoli D, Carelli S, Alberti L, Cereda C. Towards genomic-NBS: technical feasibility of NGS starting from dried blood spots. SIGU, Italian Society of Human Genetics. Rimini, Italy, October 4-6, 2023
- **Mauri A,** Alberti L, Berardo C, Biganzoli D, Iascone M, Lucchi S, Benedetti E, Carelli E, Carelli S, Pretese R, Gasperini S, Cereda C. Identification of a novel HADHB splice site variant causing Mitochondrial Trifunctional Protein Deficiency 2. SIGU, Italian Society of Human Genetics. Rimini, Italy, October 4-6, 2023

- Esposito L, **Mauri A,** Rey F, Maghraby M, Castellotti B, Zuccotti G, Tonduti D, Carelli S, Cereda C. Further insights into Allan-Herndon-Dudley syndrome: a novel SLC16A2 splice site variant. SIGU, Italian Society of Human Genetics. Rimini, Italy, October 4-6, 2023
- Carelli S, Rey F, Messa L, Maghraby E, Esposito L, **Mauri A,** Berardo C, Cereda C. Dealing with 3D culture systems: some applicative examples (pro and cons). 20th SINS congress, Turin, Italy, September 14-17, 2023
- Rey F, Esposito L, **Mauri A,** Berardo C, Zuccotti G, Tonduti D, Rosati J, Carelli S, Cereda C. Leukodystrophy -affected patients harboring mutations in Pol III gene present a profound transcriptional dysregulation. 20th SINS congress, Turin, Italy, September 14-17, 2023
- **Mauri A,** Benedetti S, Meta A, Berardo C, Carelli S, Alberti L, Cereda C. Design and set up of NGS-based newborn screening approach from dried blood spots. ESHG - European Society of Human Genetics, Glasgow, Scotland, June 10-14, 2023
- Berardo C, **Mauri A,** Messa L, Rey F, Cordaro E, Pelizzo G, Zuccotti G, Carelli S, Calcaterra V, Cereda C. Coding and non-coding transcriptomic profiles in pediatric severe obesity. ESHG - European Society of Human Genetics, Glasgow, Scotland, June 10-14, 2023
- Rey F, Esposito L, **Mauri A,** Tonduti D, Rosati J, Carelli S, Cereda C. Global transcription is profoundly impacted in primary fibroblasts obtained from pediatric patients affected by leukodystrophy harboring mutations in Pol III-encoding genes. ESHG - European Society of Human Genetics, Glasgow, Scotland, June 10-14, 2023
- Berardo C, Lucchi S, Cappelletti L, Saielli L, Pratiffi E, Fazzone S, Meta A, Benedetti E, Benedetti S, **Mauri A,** Furlan F, Menni F, Gasperini S, Rovelli V, Banderali G, Alberti L, Cereda C. A 5-year experience of newborn screening in Lombardia Region (Italy). ESHG - European Society of Human Genetics, Glasgow, Scotland, June 10-14, 2023
- Fontana L, Rondinone O, Moresco G, **Mauri A,** Peron A, Pietrogrande L, Miozzo M. A TBX4 variant in congenital patellar dislocation, an ultra-rare disorder with unknown genetic etiology. ESHG - European Society of Human Genetics, Glasgow, Scotland, June 10-14, 2023
- Rondinone O, Murgia A, Costanza J, Tabano S, Camanni M, Pesenti C, **Mauri A,** Colapietro P, Radaelli T, Ferrazzi E, Fontana L, Sirchia S, Miozzo M. Genome-wide DNA methylation profile of maternal and cord blood in spontaneous and egg-donation pregnancies. ESHG - European Society of Human Genetics, Glasgow, Scotland, June 10-14, 2023
- Berardo C, **Mauri A,** Messa L, Rey F, Cordaro E, Pelizzo G, Zuccotti G, Carelli S, Calcaterra V, Cereda C. Transcriptional profile of subcutaneous adipose tissue in relation to pediatric obesity: a pilot study. Obes Facts (2023) 16 (Suppl. 1): 1–351,

https://doi.org/10.1159/000530456. 30th European Congress on Obesity (ECO 2023), May 17-20, 2023 (IF 4.807 – 2023)

- **Mauri A,** Duse A, Palm G, Previtali R, Bova S, Olivotto S, Benedetti S, Coscia F, Veggiotti P, Cereda C. Identification of a novel SLC2A1 splice site variant causing GLUT1 deficiency syndrome. CIMED, Hybrid, March 22-24, 2023.
- **Mauri A,** Saielli LA, Alfei E, Iascone M, Marchetti D, Cattaneo E, Di Lauro A, Antonelli L, Alberti L, Bonaventura E, Veggiotti P, Spaccini L, Cereda C. Malattia di Menkes complicata da concomitante deficit di aminoacilasi 1. SIMMESN, November 9-11, 2022
- Lucchi S, Berardo C, Cappelletti L, Saielli LA, Pratiffi E, Fazzone S, Meta A, Benedetti E, Benedetti S, **Mauri A,** Furlan F, Menni F, Gasperini S, Rovelli V, Cereda C. Screening neonatale delle malattie metaboliche ereditarie: studio di 5 anni condotto sulla popolazione lombarda. SIMMESN, 9-11 November 2022
- Berardo C, **Mauri A,** Messa L, Rey F, Cordaro E, Pelizzo G, Zuccotti G, Carelli S, Calcaterra V, Cereda C. Epigenetic Impact on Pediatric Obesity: Implication of Long Non-Coding RNAs. Obesity week, November 1-4, 2022
- Rondinone O, **Mauri A,** Moresco G, Costanza J, Santaniello C, Colapietro P, D'Ursi P, Uggeri M, Marfia G, Guarnaccia L, Navone S, Manzoni G, Grilli F, Milani D, Miozzo M, Fontana L. Identification and modelling of a novel physiological LRIG2 isoform: role in Urofacial Syndrome and in motoneuron differentiation. SIGU, Italian Society of Human Genetics, Trieste, Italy, September 7-9, 2022
- **Mauri A,** Duse A, Palm G, Previtali R, Bova S, Olivotto S, Benedetti S, Coscia F, Veggiotti P, Cereda C. Novel SLC2A1 mutations in GLUT1DS pediatric patients. SIGU, Italian Society of Human Genetics, Trieste, Italy, September 7-9, 2022
- Berardo C, Lucchi S, Cappelletti L, Saielli LA, Benedetti S, **Mauri A,** Furlan F, Menni F, Gasperini S, Rovelli V, Cereda C. Newborn screening of inborn errors of metabolism: a 5-year review of Lombard population. SIGU, Italian Society of Human Genetics, Trieste, Italy, September 7-9, 2022
- Rondinone O, **Mauri A,** Moresco G, Costanza J, Santaniello C, Colapietro P, D'Ursi P, Uggeri M, Marfia G, Guarnaccia L, Navone S, Manzoni G, Grilli F, Milani D, Miozzo M, Fontana L. Identification of a novel physiological LRIG2 splicing variant associated with the development of Urofacial Syndrome. ESHG, European Society of Human Genetics, Vienna, Austria, June 11-14, 2022
- **Mauri A,** Duse A, Olivotto S, Bova S, Veggiotti P, Cereda C. Three novel SLC2A1 mutations in GLUT1DS pediatric patients. ESHG, European Society of Human Genetics, Vienna, Austria, June 11-14, 2022