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Altered Mitochondrial DNA Methylation Patterns in Thrombosis

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ABSTRACT

DNA methylation being one of the chief controllers of gene expression has not only been the reason behind the initiation of a plethora of diseases like Cancer, Alzheimer's disease, Parkinson's disease, etc. But is an active contributor to the pathophysiology of several cardiovascular diseases like coronary artery disease (CAD), Atherosclerosis, Stroke, Cardiomyopathy, etc. The role of nuclear DNA methylation in VTE has been studied earlier, but the comparison of methylation in both nuclear genes and mitochondrial genes in high altitude VTE (HA-VTE) and sea level VTE (SL-VTE) patients has not been studied in depth. Through this present study, DNA methylation patterns of mitochondrial encoded as well as nuclear-encoded mitochondrial genes of five high-altitude VTE patients and five sea-level VTE patients have been obtained. On comparing HA-VTE and SL-VTE methylation, one hundred and twenty hypermethylated genes and one hundred and thirty-eight hypomethylated genes were observed. Post gene enrichment and ontology study, the TCA cycle and NADH dehydrogenase were found to be the highly enriched pathways in both the study groups. Protein-protein interaction network using STRING pointed out the enriched pathway of L-2-hydroxyglutaric acid when both the gene sets were enriched. These results show the crucial role of mitochondrial DNA methylation in the pathophysiology of thrombosis and show great potential to study the role of mitochondria in thrombosis.

Keywords: DNA methylation; Mitochondria; Venous Thromboembolism; Hyper and hypo methylation

1. INTRODUCTION

Pulmonary embolism (PE) and Deep vein thrombosis (DVT) both represents different expressions of venous thromboembolism (VTE)[1]. VTE is the third most prevalent cardiovascular condition, with a 1 to 3 in 1,000 annual incidence rate[2]. Medical studies have demonstrated that the development of VTE is influenced by both hereditary and environmental variables[3-6]. The acquired risk factors for VTE, which include trauma, major surgery, pregnancy, and cancer, are equally as well-known as the hereditary risk factors for the condition[7-8]. Over 40% of individuals with Venous thromboembolism (VTE) are believed to possess at least one inherited or acquired risk factor, contributing to the occurrence of the thrombotic event[6-9]. Nonetheless, approximately half of VTE patients, who lack apparent risk factors, experience what is referred to as unprovoked VTE[10].

Epigenetic mechanisms, including DNA methylation, activity of non-coding RNA and histone modification, regulate gene transcription and maintain genomic stability without making alterations to the DNA sequence itself. Without affecting DNA sequence, epigenetic mechanisms such as DNA methylation, histone modification, and non-coding RNA activity control gene transcription and genomic stability[11-12]. Environmental modifications like diet, stress, inflammation, exercise, and drug use have an impact on epigenetics. There was a belief that the environment accounted for about 80% of the risk of human disease[12-13]. The relationship between genes and environment may therefore be mediated by epigenetic mechanisms, which would account for the significance of the epigenome in disease pathophysiology.

DNA methylation involves the enzymatic attachment of a methyl group to the fifth carbon position of cytosine within the sequence 5-cytosine-guanosine (CpG) during the post-synthetic phase of DNA synthesis[14-15]. DNA methylation in mammalian cells primarily affects the cytosine residues of CpG dinucleotides. The majority of the genome's CpG dinucleotides—including endogenous repeats, gene bodies and transposable elements—are methylated to a degree of up to 70%[15]. Numerous diseases' etiologies involve abnormal DNA methylation. CpGs in non-promoter regions are often methylated in healthy cells, but CpG islands in promoter areas are typically hypomethylated. Because transcriptional inhibition in normally silent regions is weakened, transcription can start at the incorrect place as a result of global hypomethylation of non-promoter regions of DNA, which can lead to instability and structural abnormalities in chromosomes[16].

Additionally, this leads to the excessive or potentially dangerous expression of genes that are usually silenced. The inactivation of protective or disease-suppressor genes, allelic loss, and gene mutation may be made easier by hypermethylation of CpG islands in gene promoter regions[17]. The entire level of DNA methylation across

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the genome is represented by blood-based global DNA methylation. One of the initial chemical alterations in a cell's transformation from a healthy to a sick condition, global DNA methylation has received a lot of interest recently as a biomarker for various illnesses, which includes type 2 diabetes, cancer and cardiovascular disease[16].

The processes of mtDNA epigenetic control, however, have received less research than those of nuclear DNA epigenetic regulation, and there has only been a recent increase in interest in this area[18]. Since mtDNA lacks histones and differs structurally from nuclear chromatin, DNA methylation is one of the most researched epigenetic alterations that occur in this molecule[19]. In the area of several cardiovascular diseases, Mitochondrial DNA methylation signatures have been previously studied. In a research study involving an analysis of platelet mitochondria from 10 patients with cardiovascular disease (CVD) and 17 healthy individuals, it was observed that CVD patients exhibited increased methylation of the MT-CO1, MT-CO2, MT-CO3, and MT-TL1 genes[20]. This study presented the initial evidence suggesting a possible involvement of mitochondrial DNA (mtDNA) methylation dysfunction in the development of cardiovascular diseases (CVDs).

Although no such studies have been reported to date that depict a connection between VTE and mtDNA methylation of high-altitude VTE patients in comparison to sea-level VTE patients, this present study is a first of its kind that presents a detailed mtDNA methylation scenario between the above-mentioned groups to understand the mtDNA methylation pattern.

2. MATERIALS AND METHOD

2.1 Study Subjects

The current study was carried out in compliance with the ethical principles of the Indian Council of Medical Research. Study volunteers were Indian Army soldiers who were in two study groups: VTE patients and healthy subjects from the Army Research and Referral Hospital (R & R), Delhi, India (for patients with VTE at sea level), and the Western Command Hospital, Chandi Mandir, Chandigarh (for patients with VTE at high altitude) [Fig. 1]. Patients with a history of systemic disease, any previous surgeries, vasculitis, or cancer were not included in the study. Additionally, at the time of sample collection, the clinical profile of the patients, the time and location of the venous thrombosis (VT) episode, and the presence of predisposing variables (such as surgery, trauma, prolonged immobilization, hypertension, diabetes, and familial history of bleeding) were reported. All patients had their VTE diagnosis confirmed by at least one radiological imaging technique, such as magnetic resonance imaging, contrast-enhanced computed tomography, color Doppler, or computed tomography angiography.

2.2 Extraction of DNA & Quantification

DNA was extracted from peripheral blood. Using the Nanodrop Spectrophotometer (Thermo Scientific; 2000) and the Qubit dsDNA HS test kit (Thermo Fischer Scientific), the concentration and purity of DNA samples were measured. Agarose gel electrophoresis was used to examine the integrity of the DNA. Samples with the best yield and concentration that passed quality testing were declared eligible for creating Illumina libraries.

2.3 Library Preparation

Methylation sequencing libraries were created using the SureSelectXT Methyl-Seq Target Enrichment System for Illumina Multiplexed Sequencing (Agilent Technologies, Santa Clara, CA, USA). In brief, 400–500ng of genomic DNA were sheared with a Covaris S2 sonicator (Covaris, Woburn, Massachusetts, USA) to produce around 150–200 bp-sized fragments. Here, Agilent Technologies, Palo Alto, California, USA, used D1000 DNA screen tapes and chemicals to check the fragment size distribution on an Agilent 2200 TapeStation. According to the SureSelectXT Methyl-Seq Target Enrichment methodology, the fragments were end-repaired, adenylated, and ligated to Illumina adaptors. Illumina Universal Adapter: CCCTACACGACGCTCTTCCGATCT-3' and Index Adapter: 5'-GATCGGAAGAGCACACGTCTGAACTCCAGTCAC were the adapters utilized in the investigation. [INDEX] ATCTCGTATGCCGTCTTCTGCTTG-3'. [INDEX] - Special sequence to recognize sample-specific information. With the use of Sera-MagTM Select beads (Cytiva, # 29343057), the adapter-ligated DNA was cleaned. Using the SureSelect custom capture Library (Agilent Technologies # G9496C) and the SureSelectXT Methyl-Seq Target Enrichment procedure, the ligated product was then maintained for hybridization. Using Sera-mag speedBead blocked streptavidin particle (Cytiva, #CAT 21152104010150), hybridized library fragments were extracted by magnetic capture. After being eluted in 20 l of SureSelect Elution Buffer, the collected library was converted to bisulfite using the EZ DNA Methylation Gold Kit. Here, uracil residues are formed from unmethylated cytosine residues in the library, whereas methylated cytosine residues are left unaltered. Desulphonation is followed by PCR amplification of the treated DNA, which uses 10-8 PCR cycles to convert uracil residues in the sample to thymidine. PCR product cleanup was carried out using Sera-MagTM Select beads.

Using eight PCR cycles, the modified collected DNA library was amplified by PCR indexing. With Sera-MagTM Select beads (Cytiva, # 29343057), the final PCR products (sequencing libraries) were purified. Qubit fluorometer was used to quantify and validate captured libraries, and Agilent Tape Station was used to run an aliquot. The samples' Illumina-compatible sequencing libraries had enough concentration and an average fragment size of 374 bp to produce the requisite sequencing results.

2.4 Sequencing and Alignment

Following the manufacturer's recommendations, the libraries were paired-end sequenced for 150 cycles using an Illumina Novaseq 6000 sequencer (Illumina, San

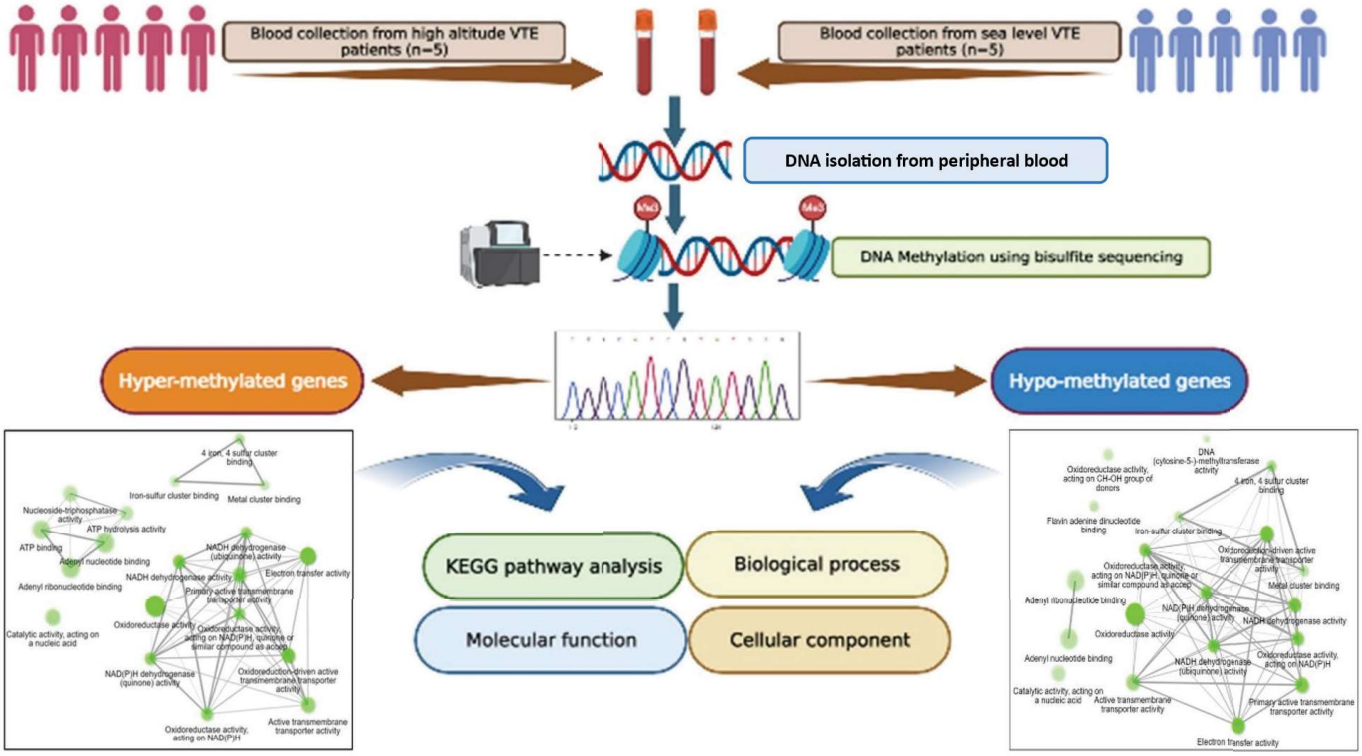

Figure 1. Schematic Representation of the study.

Diego, USA). Using the Bcl2fastq 1 software version 2.20, the data received from the sequencing run was de-multiplexed, and FastQ files were produced based on the distinctive dual barcode sequences. Bases above Q30 were taken into consideration after the adapter sequences were cut. During read pre-processing, low-quality bases were filtered off and used for downstream analysis.

2.5 Data Analysis

Using the FastQC 2 v0.11.32 tool, the raw Illumina readings from samples were quality-checked. Using the Trimgalore v0.4.0 3 tool, raw readings were trimmed to have a minimum quality score of 30 and a minimum length of 20. These cleaned-up reads were utilized to execute a Bismark 4 alignment against the hg19 version of the human genome, and the alignments were then used for a Bismark methylation extraction. Using the methylKit 5 (R package) program, the collected CpG context was utilized to identify areas with various levels of methylation. With the aid of Homer 6, the CpG context was annotated.

2.6 Identification of Differential Methylated Regions and Positions

The methylKit (R package) was used to process methylation call files or CpG reports to identify bases that were differentially methylated across samples. The readings on both strands of a CpG dinucleotide from the samples were combined for further analysis by the desired combination of interest. Base-pair sites that are covered by both samples served as the basis for comparisons. Based on the q-value and percent methylation difference cut-offs (by default, we have chosen percent methylation difference >10%), we can choose the differentially methylated bases. The outcomes have been presented in Excel as hypo- or hyper-methylated and significantly methylated (q- value=0.01) bases per chromosome. Annotations were made using the Homer tool. The TSS (transcription start site), TTS (transcription termination site), Exon (Coding), 5' UTR Exon, 3' UTR Exon, Intronic, non-coding, or Intergenic area are the most frequently identified annotations.

3. RESULTS

In this present study, the status of methylation in 233 targeted genes was studied. Later on, both hypermethylation and hypomethylation results were assessed between two groups, i.e., High altitude VTE Patients and Sea level VTE Patients. The number of genes getting hypermethylated when compared between high altitude patient group vs sea level patient group is 120, and the number of genes getting hypomethylated when compared between sea high altitude patient group vs. sea level patient group is 138. Further, these genes were assessed for enrichment studies and Gene ontology values using the software ShinyGO 0.77, and STRING was used for protein-protein interaction (PPI).

Hypermethylation in High Altitude Patient Groupvs. Sea Level Patient Group

In the high-altitude patient group vs. Sea level patient group, a total of 120 genes were hypermethylated throughout the genome. Table 1 shows the distribution of these hypermethylated genes from chromosome 1 to chromosome X. A Graphical representation of the no. of genes distributed from chromosome 1 to chromosome X in hypermethylation is depicted in Fig 2.

Table 1. Distribution of hypermethylated genes under each chromosome in group B vs. group A

Sl. no.	Chromosome	Hypermethylated genes
1	1	ATAD3A, ATAD3B, ATAD3C, TMEM240, MFN2, C1orf195, SDHB, WARS2, MSTO2P, NAXE, SDHC, FASLG, RABGAP1L, MIR215, COQ8A, COA6
2	2	RNASEH1, DNMT3A, MIR1301, MDH1, HSPD1, MARS2, NDUFS1, BCS1L, MFF, MIR4786, D2HGDH, GAL3ST2
3	3	PRKAR2A-AS1, ACAD9, SNORA58, MRPS22, LXN, LINC00969
4	4	PPARGC1A, LOC101928314, SLC25A4
5	5	NDUFS6, OXCT1-AS1, NDUFAF2, SV2C, LOC553103, HARS, LOC644762
6	6	FARS, LOC101927972, MTO1, SMIM8, RTN4IP1, LOC105377962, ZBTB2
7	7	MRM2, GARS, TRG-AS1, LINC01446, 2NF733P
8	8	DEFB4B, NDUFB9
9	9	TXN, MRPS2, LOC101928525
10	10	DNA2, MRPS16, TWNK, LZTS2, SFXN4
11	11	C11orf21, WEE1, NDUFV1, SDHD, FOXRED1
12	12	NDUFA9, MFAP5, DNM1L, YARS2, C12orf65
13	13	Ankrd26p3 (Pseudo), NAXD
14	14	NUBPL, APOPT1
15	15	MIR6766, POLG
16	16	COQ7, MIR4721, COX6A2, COX4I1, RPL13
17	17	SCO1, ELAC2, ATPAF2, MIEF2, COASY, MRPL12, SLC25A10, TBCD
18	19	ATP5D, NDUFS7, PET100, EIF3G, GTPBP3, TMEM161A, COQ8B, VASP, 2NF818P, NDUFA3
19	20	IDH3B, NDUFAF5, ATP5E
20	22	POLR3H, NDUFA6-AS1, TRMU, RNA5-8S5
21	X	PDK3, NDUFB11, HSD17B10, ABCB7, COX7B, TIMM8A, AIFM1, DNASE1L1

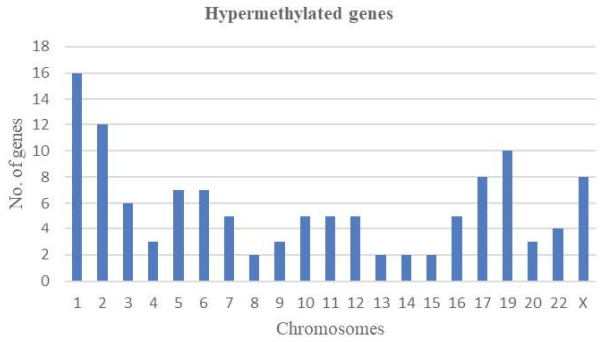

Figure 2. Graphical representation of the no. of genes distributed from chromosome 1 to chromosome X in hypermethylation scenario.

Further analysis of these 120 genes was carried out regarding gene ontology and enrichment studies using ShinyGO 0.77 software. Also, protein-protein interaction (PPI) studies were carried out using STRING database post-gene enrichment studies. To understand the network of hypermethylated genes amongst themselves, KEGG pathway results were analyzed first. Table 2 demonstrates the top 10 KEGG pathways, their fold enrichment score, Enrichment FDR, no. of genes, and number of genes found in each of these pathways respectively.

Table 3 shows the highly enriched pathways with Enrichment FDR, the number of genes, pathway genes, and Fold enrichment scores. Ten highly enriched pathways

Table 2. Top 10 KEGG pathway enriched according to fold enrichment from the hypermethylated gene interactions.

Enrichment FDR	No. of genes	Pathway genes	Fold Enrichment	Pathways
7.3E-07	5	30	43.7	Citrate cycle (TCA cycle)
9.0E-14	13	134	25.4	Oxidative phosphorylation
4.0E-14	14	155	23.7	Non-alcoholic fatty liver disease
1.7E-02	2	23	22.8	Proximal tubule bicarbonate reclamation
4.8E-14	15	203	19.4	Diabetic Cardiomyopathy
1.8E-15	17	232	19.2	Thermogenesis
3.7E-03	3	44	17.9	Aminoacyl Trna biosynthesis
2.2E-12	14	223	16.4	Chemical carcinogenesis
1.6E-12	15	266	14.8	Parkinson disease
2.4E-11	14	272	13.5	Prion disease

Table 3. Collective representation of gene ontology in biological process, molecular function and cellular component, the pathways involved and the fold enrichment values associated with this

GO (Biological Process)				
Enrichment FDR	**nGenes**	**Pathway genes**	**Fold enrichment**	**Pathways enriched**
3.5E-17	14	82	44.7	NADH dehydrogenase complex assembly
3.5E-17	14	82	44.7	Mitochondrial respiratory chain complex I assembly
9.3E-08	7	51	36	Mitochondrial RNA metabolic process
7.9E-18	16	119	35.2	Mitochondrial respiratory chain complex assembly
6.8E-16	15	131	30	Respiratory electron transport chain
8.6E-14	13	114	29.9	ATP synthesis coupled electron transport
1.2E-12	12	106	29.7	Aerobic electron transport chain
2.4E-12	12	113	27.8	Mitochondrial ATP synthesis coupled electron transport
8.1E-07	7	70	26.2	Mitochondrial electron transport, NADH to ubiquinone
1.3E-14	16	200	21	Electron transport chain

GO (Molecular Function)				
Enrichment FDR	**nGenes**	**Pathway genes**	**Fold enrichment**	**Pathways enriched**
6.8E-09	8	62	33.8	NADH dehydrogenase activity
1.7E-07	7	60	30.6	NADH dehydrogenase (ubiquinone) activity
1.8E-07	7	64	28.7	NAD(P)H dehydrogenase (quinone) activity
1.6E-05	5	46	28.5	4 iron, 4 sulphur cluster binding
4.9E-10	10	100	26.2	Oxidoreductase-driven active transmembrane transporter activity
5.2E-07	7	77	23.8	Oxidoreductase activity, acting on NAD(P)H
3.4E-13	14	158	23.2	Electron transfer activity
2.9E-07	8	109	19.2	Oxidoreductase activity, acting on NAD(P)H
1.3E-04	5	73	17.9	Iron-sulphur cluster binding
1.3E-04	5	73	17.9	Metal cluster binding
GO (Cellular Components)				
Enrichment FDR	**nGenes**	**Pathway genes**	**Fold enrichment**	**Pathways enriched**
1.8E-06	3	4	196.5	Mitochondrial respiratory chain complex II, succinate dehydrogenase
1.2E-20	16	101	41.5	Respiratory chain complex
6.9E-19	15	102	38.5	Mitochondrial respirasome
1.3E-19	16	117	35.8	Respirasome
2.7E-07	6	47	33.4	Nucleoid
2.7E-07	6	47	33.4	Mitochondrial nucleoid
1.9E-09	8	66	31.8	Mitochondrial respiratory chain complex-I
1.9E-09	8	66	31.8	NADH dehydrogenase complex
1.9E-09	8	66	31.8	Respiratory chain complex-I
1.6E-12	12	132	23.8	Oxidoreductase complex

of biological processes, molecular functions, and cellular compartments are mentioned.

The correlation between the significant routes in biological processes shown in the enrichment tab is summarized in Fig. 3A. Pathways that share a lot of genes tend to group. Significant P-values are shown by larger dots. The significant molecular functions have been shown through the bar chart presentation (Fig. 3B). The Top 10 molecular functions were enriched which has been mentioned in Table 3. Out of these, the top 5 highest enriched molecular functions based on their fold enrichment scores are mentioned in Fig 3B with a gradient of coloring. Blue is the highest score and orange is the lowest score.

Lastly, the cellular components have been shown using the network mode of demonstration (Fig. 3C). The component with the darkest node is the Mitochondrion. The other enriched components although not as dark as the mitochondria are the Mitochondrial envelope and organelle inner membrane.

Similar to gene enrichment and ontology studies, Protein-protein network (PPI) studies were carried out using STRING online software. An interactive protein network was generated from the input proteins (Fig. 4). The top 5 pathways to get enriched are mentioned in Table 4.

Hypomethylation in High Altitude Patient Group vs. Sea Level Patient Group

When the hypomethylation was compared between High-altitude patients vs. Sea level patients, a total of 138 genes were retrieved. These genes were further distributed from chromosome 1 to chromosome X (Fig. 5). Table 5 shows the number of genes with gene names under each respective chromosome.

Post-gene enrichment analysis of these 138 genes generated a KEGG pathway that enriched the top 10 highly significant pathways (Table 6).

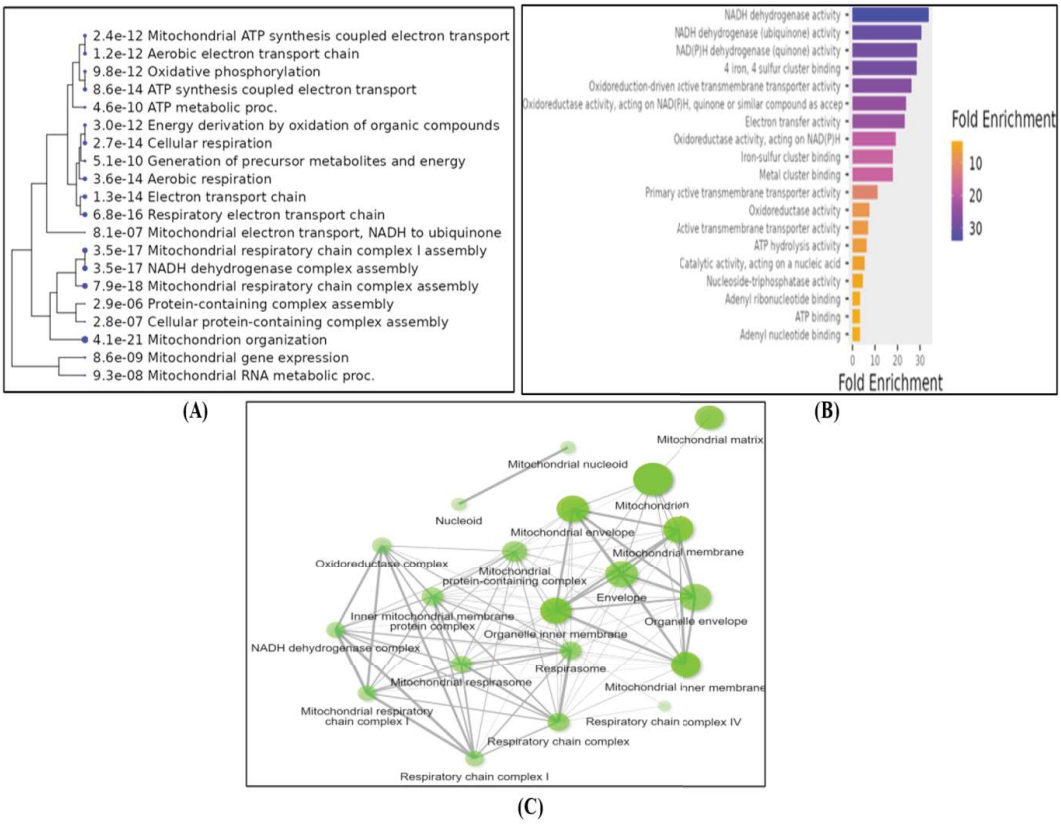

Figure 3. Gene enrichment analysis of the hypermethylated genes. Gene enrichment analysis of the hypermethylated genes. (A) The hierarchical pathway correlation amongst of the biological functions amongst each other. Bigger dots indicate more significant p-values. (B) The bar chart presentation of the important molecular functions highlighted post enrichment analysis. hierarchical pathway correlation amongst each other. (C) The cellular component networks between the highly overlapped gene sets.

The hierarchical tree demonstrates the highly enriched biological functions and the correlation amongst other biological functions (Fig. 6A). Also, the most significant pathways found through the hierarchical tree are aerobic respiration and cellular respiration with bigger nodes compared to the other functions.

In molecular function, DNA (cytosine-5-)-methyltransferase activity is highly enriched amongst the top 10 functions with a Fold Enrichment score of 149 (Fig. 6B).

Lastly, the cellular components using network mode have highlighted Mitochondrion with the darkest shade amongst the other cellular components as the highest enriched component. (Fig. 6C).

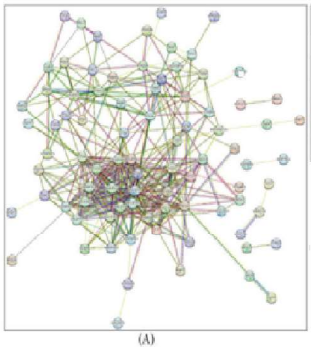

Figure 4. Protein-protein interaction (PPI) network using STRING.

Lastly, the Protein-Protein interaction study of the hypomethylated genes performed and the top 5 pathways to get enriched are mentioned in Table 8. L-2-hydroxyglutaric aciduria, metabolic repair, Ubiquinone biosynthesis, and NADH dehydrogenase complex were the highly enriched STRING pathways in the hypomethylation gene sets with the strength of 1.94, 1.68, 1.6, 1.59 and 1.59 respectively. The PPI network is shown in Fig. 7.

4. DISCUSSION

The gene enrichment and ontology analysis of the present study of both hypomethylated as well as hypermethylated genes have repeatedly highlighted the TCA cycle, as well as the NADH dehydrogenase pathway in association with the patient population of high altitude and sea level. Being interconnected to each other for the proper functioning of energy metabolism, the disruption in their function has been the cause of a plethora of CVDs.

As a crucial factor in the initiation of the TCA cycle, any significant alterations in metabolites connected to the tricarboxylic acid (TCA) cycle are brought on by changes

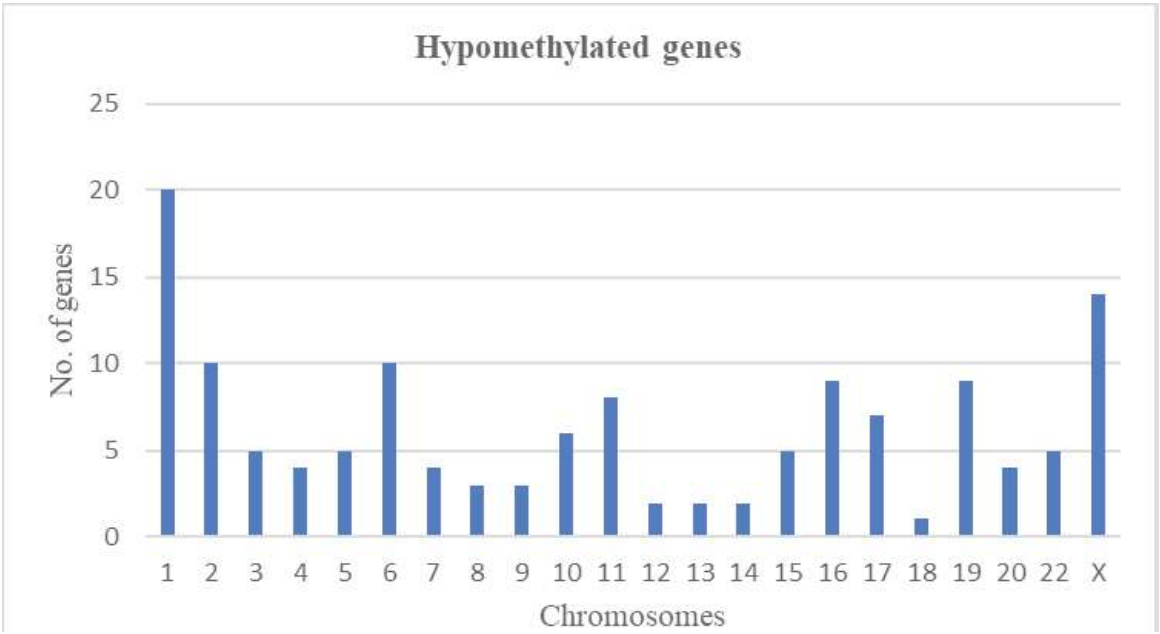

Figure 5. Graphical representation of the no. of genes distributed from chromosome 1 to chromosome X in hypomethylation scenario.

Table 4. Top five STRING enriched pathways of hypermethylation genes

Local Network Custer (STRING)					
Sl. no.	**Cluster**	**Description**	**Count in network**	**Strength**	**False discovery rate**
1.	CL.12040	L-2-hydroxyglutaric aciduria & metabolic repair	3 of 6	1.99	0.0021
2.	CL.11690	Fumarate reductase complex & Mitochondrial DNA depletion syndrome 5	3 of 6	1.99	0.0021
3.	CL.13387	NADH dehydrogenase activity	3 of 7	1.93	0.0025
4.	CL.13351	NADH dehydrogenase activity	3 of 8	1.87	0.0033
5.	CL. 13844	Ubiquinone biosynthesis	3 of 11	1.73	0.0068

Table 5. Distribution of hypomethylated genes under each chromosome in high altitude patient vs. Sea level patient

Sl. no.	Chromosome	Hypomethylated genes
1	1	ATAD3A, ATAD3B, ATAD3C, TMEM240, C1orf195, ATP13A2, PARS2, DAB1-AS1, LINC01781, MSTO2P, NAXE, PPOX, NDUFS2, CFAP126, AXDND1, ACBD3, COQ8A, COA6, GPR137B, FH
2	2	RNASEH1, DNMT3A, GAREM2, HADHA, NDUFS1, MDH1B, MFF, MIR4786, D2HGDH, GAL3ST2
3	3	ACAD9, AHSG, OPA1-AS1, LINC00969, LOC220729
4	4	PPARGC1A, CXCL11, NDUFC1, C4orf46
5	5	NADK2, NDUFAF2, FCHO2, LOC553103, NHP2
6	6	LOC101927950, LOC101927972, UQCC2, FGD2, TCTE1, AARS2, MTO1, SMIM8, RTN4IP1, TBC1D32
7	7	MRM2, LINC01446, MDH2, LOC105375401
8	8	CSMD1, POTEA, PDP1
9	9	JAK2, NDUFA8, LOC101928525
10	10	ARMC4P1, RUFY2, CUTC, TWNK, USMG5, XPNPEP1

11	11	EHF, LBHD1, NDUFV1, DOC2GP, MIR7113, MIR4691, FAT3, FOXRED1
12	12	NDUFA9, PUS1
13	13	ANKRD26P3, NAXD
14	14	NUBPL, APOPT1
15	15	GOLGA6L2, TYRO3, TYROP, MFGE8, POLG
16	16	NME3, SNORA10, COQ7, COX-6A2, LINC01571, COQ9, KARS, SPG7, RPL13
17	17	TIMM22, ATPAF2, MIEF2, LOC105371703, GGA3, MRPL12, SLC25A10
18	18	AFG3L2
19	19	ATP5D, NDUFS7, NDUFA11, EIF3G, TMEM161A, ECH1, VASP, OPA3, ZNF818P
20	20	IDH3B, COX4I2, DNMT3B, MAPRE1
21	22	ISX-AS1, ACO2, POLR3H, TRMU, SCO2
22	X	HCCS, PDHA1, PDK3, NDUFB11, RBM10, HSD17B10, UQCRBP1, COX7B, TIMM8A, RNF113A, AIFM1, PDZD4, TAZ, DNASE1L1

Table 6. Top 10 highly enriched KEGG pathways from the hypomethylated gene interactions

Enrichment FDR	No. of genes	Pathway genes	Fold Enrichment	Pathways
1.9E-06	5	30	37.2	Citrate cycle (TCA cycle)
1.7E-02	2	19	23.5	2-Oxocarboxylic acid metabolism
7.0E-10	11	134	18.3	Oxidative Phosphorylation
2.5E-09	11	155	15.9	Non-alcoholic fatty liver disease
3.4E-10	13	203	14.3	Diabetic cardiomyopathy
7.4E-03	3	47	14.3	Pyruvate metabolism
2.3E-10	14	232	13.5	Thermogenesis
8.3E-03	3	50	13.4	Cysteine & methionine metabolism
2.8E-06	8	148	12.1	Retrograde endocannabinoid signaling
9.7E-08	11	223	11	Chemical carcinogenesis

Post gene ontology studies, 10 highly enriched pathways of biological process, molecular functions, and cellular compartments are mentioned in Table 7.

Table 7. Collective representation of the pathways and fold enrichment values involved in biological processes, molecular function, and cellular components from the hypomethylated gene interactions.

GO (Biological Process)				
Enrichment FDR	**nGenes**	**Pathway Genes**	**Fold Enrichment**	**Pathways**
2.1E-16	14	82	38.2	NADH dehydrogenase complex assembly
2.1E-16	14	82	38.2	Mitochondrial respiratory chain complex I assembly
1.2E-06	6	36	37.2	Tricarboxylic acid cycle
5.4E-17	16	119	30	Mitochondrial respiratory chain complex assembly

3.1E-09	9	70	28.7	Mitochondrial electron transport, NADH to ubiquinone
2.1E-14	14	114	27.4	ATP synthesis coupled electron transport
2.1E-16	16	131	27.3	Respiratory electron transport chain
1.0E-05	6	51	26.3	Mitochondrial RNAetabolic process
5.1E-13	13	113	25.7	Mitochondrial ATP synthesis coupled electron transport
7.1E-12	12	106	25.3	Aerobic electron transport chain

GO (Molecular Process)

Enrichment FDR	nGenes	Pathway Genes	Fold Enrichment	Pathways
1.1E-03	2	3	149	DNA (cytosine-5)-methyltransferase activity
2.9E-08	8	62	28.8	NADH dehydrogenase activity
5.9E-07	7	60	26.1	NADH dehydrogenase (ubiquinone) activity
7.2E-07	7	64	24.4	NAD(P)H dehydrogenase (quinone) activity
5.7E-05	5	46	24.3	4iron, 4 sulphur cluster binding
4.3E-09	10	100	22.3	Oxidoreduction-driven active transmembrane transporter
1.9E-06	7	77	20.3	Oxidoreductase activity, acting on NAD(P)H, quinone or similar compound as acceptor
1.2E-06	8	109	16.4	Oxidoreductase activity, acting on NAD(P)H
1.5E-08	11	158	15.6	Electron transfer activity
4.7E-04	5	73	15.3	Iron-sulphur cluster binding

GO (Cellular Component)

Enrichment FDR	nGenes	Pathway Genes	Fold Enrichment	Pathways
3.3E-05	4	22	40.6	Respiratory chain complex IV
2.4E-10	9	66	30.5	Mitochondrial respiratory chain complain I
2.4E-10	9	66	30.5	NADH dehydrogenase complex
2.4E-10	9	66	30.5	Respiratory chain complex I
2.2E-14	13	101	28.8	Respiratory chain complex
8.5E-07	6	47	28.5	Nucleoid
8.5E-07	6	47	28.5	Mitochondrial nucleoid
6.7E-13	12	102	26.3	Mitochondrial respirasome
1.3E-13	13	117	24.8	Respirasome
1.2E-11	13	168	17.3	Inner mitochondrial membrane protein complex

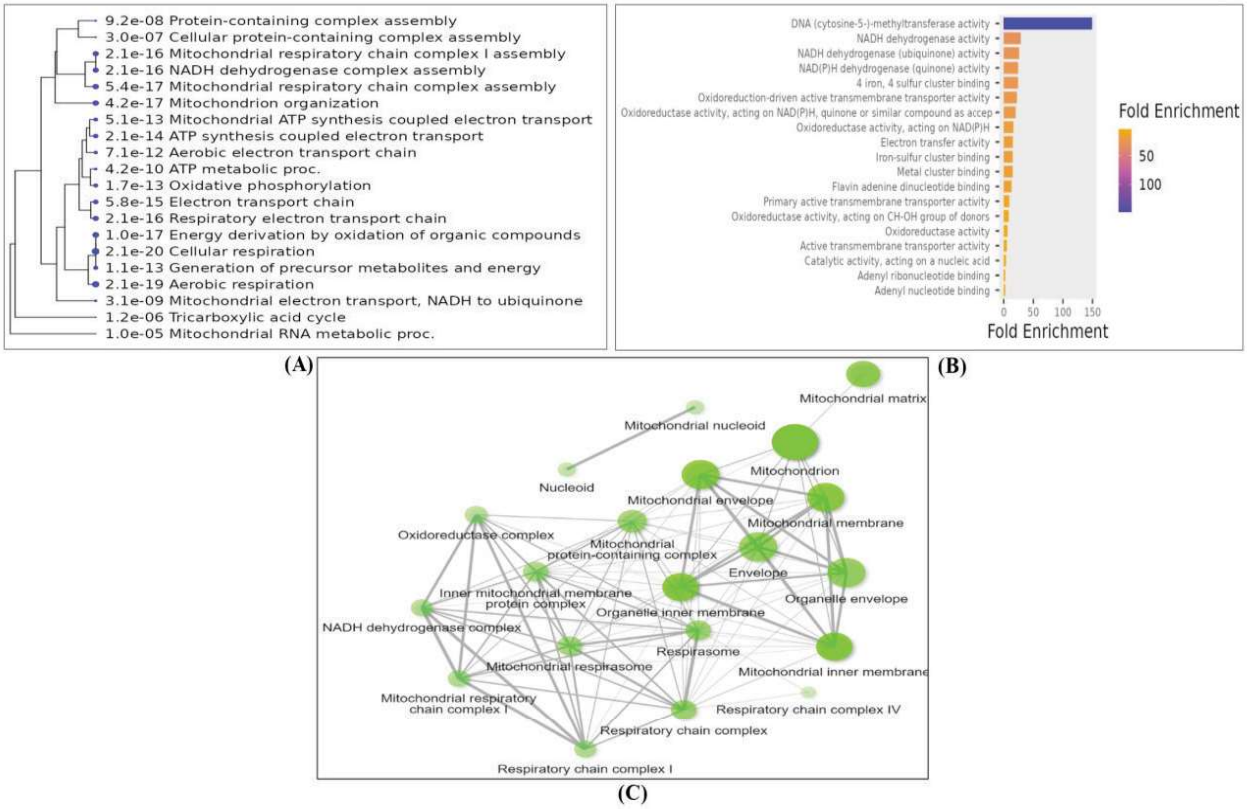

Figure 6. Analysis of the hypomethylated genes enrichment. Gene enrichment analysis of the hypomethylated genes. (6A) The hierarchical pathway correlation amongst of the biological functions amongst each other. Bigger dots indicate more significant p-values. (6B) The bar chart presentation of the important molecular functions highlighted post enrichment analysis. hierarchical pathway correlation amongst each other. (6C) The cellular component networks between the highly overlapped gene sets.

in the contractile and hemodynamic responses, as well as hypoxia and reduced energy supply. Citrate, the first molecule generated from various energy sources after acetyl coenzyme A, has been linked to cardiovascular mortality[21]. Heart Failure (HF) patients have previously been observed to have higher levels of circulating -ketoglutarate than controls[22]. L-2-hydroxyglutarate is converted by hydroxyglutarate dehydrogenase into -ketoglutarate under hypoxic conditions to counteract the reductive stress caused in the mitochondria and to restock the TCA cycle for its cataplerotic function [23]. Alpha-ketoglutarate, succinate, and malate were linked to cardioembolic stroke and atrial dysfunction in a cross-sectional investigation of acute stroke patients, but only succinate was linked to left atrial enlargement and stroke recurrence[24]. In our research, we discovered a statistically significant correlation between elevated plasma concentrations of citrate, aconitate, succinate, and malate and a greater incidence of AF. All other metabolites, except aconitate, were also found to be internally replicated. VTE being the third most occurring CVD after Stroke and Myocardial Infarction (MI), may show the consequences arising from having a troubled TCA cycle and its intermediates, which have already been documented in other CVDs and all these reports are in sync with the present mitochondrial DNA methylation outcome.

Increased aconitate, isocitrate, and malate levels were consistently associated with an increased incidence of HF. In this regard, higher citrate levels have been linked to a higher risk of death from all causes, including cancer and other nonvascular disorders[21]. Malic acid and -ketoglutaric acid effectively distinguished between patients with and without coronary lesions in a different cohort of 223 patients, 172 of whom had extensive coronary atherosclerosis and the remaining patients revealing clinical symptoms such as chest discomfort, angina, and HF [25]. Similar to this, patients with myocardial infarction had increased plasma concentrations of succinate and malate [26]. Higher citrate levels may also disrupt the formation of Acetyl CoA production, without which the entire ETC may face an energy shortage. Also, higher citrate level in the body has been connected with Heart Failure. All these scientific outcomes of previous reports support the present study findings and have a possible association with the pathophysiology of VTE.

The other pathway to be highly enriched other than the TCA cycle was the NADH dehydrogenase pathway. NADH Dehydrogenase being the Complex-I of the OXPHOS

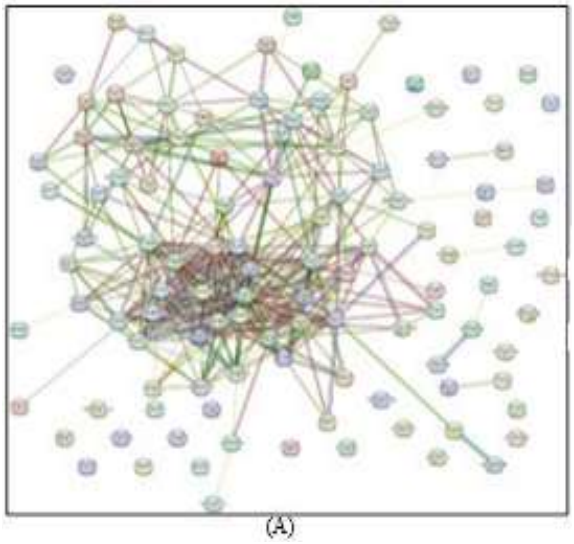

Figure 7. Protein-protein interaction (PPI) network using STRING

unit is responsible for the proper relay of electrons for the formation of ATP. TCA cycle and OXPHOS being connected through metabolic exchanges makes it very important for proper cellular functioning. One study examined the pathophysiology of the connection between stroke and NDUFC2, one of the NADH dehydrogenase complex subunit genes [27]. The Japanese-style stroke-permissive diet (JD) was used to create the stroke-prone spontaneously hypertensive rat (SHRSP) model, which was used to explore the effects of NDUFC2 disruption in the stroke condition. NDUFC2 disruption changed Complex I assembly and activity, decreased ATP levels, and altered mitochondrial membrane potential[28]. Additionally enhanced inflammation both in vivo and in vitro, as well as ROS generation[29]. After seven weeks of a Japanese-style liberal diet (JD), the animal stroke model's incidence of stroke increased to 100%. Additionally, it was shown that NDUFC2 downregulation predisposes to an increase in stroke incidence and is sufficient to cause several cellular abnormalities[30]. Considered to be the primary ROS-producing location in mitochondria is Complex I. DNA from the mitochondria and the nucleus both code for subunits of complex I. Increased mitochondrial ROS generation is caused by mutations in complex I genes[31].

Oxidative stress due to the abnormal functioning in NADH dehydrogenase resulted in mitochondrial dysfunctions through altered MMP. Hence, Stroke being a result of improper NADH functioning is a good indication of the threat towards the causation of VTE. NADH dehydrogenase genes getting methylated in several crucial genetic locations in the present study which is having agreement with the previous reports. In an ischemia-reperfusion mouse model, Chouchani, *et al*. demonstrated that the specific buildup of the citric acid cycle intermediate succinate serves as a consistent biochemical indicator of ischemia in various organs, including the heart[32]. They found that the presence of fumarate, aspartate, and malate reduced the conversion of succinate dehydrogenase (SDH) to fumarate, resulting in elevated levels of succinate[33].

Apart from the interferences caused by metabolite imbalance, the role of the genes that control the TCA cycle was also checked. MDH1 is one of the four crucial genes that regulate the TCA cycle. To see the change of expression of MDH1 gene in coronary heart disease (CHD), this study was carried out in 60 CHD patients and 60 healthy people [34].Clinical data showed that the MDH1 gene's expression was reduced in the peripheral blood cells of Coronary Heart Disease (CHD) patients. This alteration in expression could potentially result in a systematic disturbance of lipid and glucose metabolism, both of which are risk factors for CHD. This approach may serve as a biomarker for identifying individuals at risk of CHD, enabling early detection and intervention to reduce the morbidity of CHD [34]. The downregulated expression of this gene along with the irregularities in the lipid and glucose metabolism may also play a role in the causation of VTE. The present study shows the presence of the MDH1 gene as one of the top five high-level GO categories in the hypermethylated segment and depicts relevance to previously published reports.

Under the present study, the gene ontology result of the molecular function of the hypomethylated genes showed DNMT activity as the most dominant one. DNA methyltransferase plays a central role in initiating DNA

Table 8. Top five STRING enriched pathways of hypomethylation genes

Local Network Custer (STRING)					
Sl. no.	**Cluster**	**Description**	**Count in network**	**Strength**	**False discovery rate**
1.	CL.12040	L-2-hydroxyglutaric aciduria & metabolic repair	3 of 6	1.94	0.0042
2.	CL.13844	Ubiquinone biosynthesis	3 of 11	1.68	0.0149
3.	CL.13345	NADH dehydrogenase complex	8 of 35	1.6	6.82e-08
4.	CL.13347	NADH dehydrogenase complex	6 of 27	1.59	1.32e-05
5.	CL.13849	NADH dehydrogenase complex	4 of 18	1.59	0.0025

methylation by facilitating the transfer of a methyl group from SAM (S-adenosylmethionine) to the fifth position of cytosine residues in DNA [35]. With DNA methyltransferase as the point of initiation, DNA methylation has left its footprint throughout several cardiovascular diseases like CAD, Atherosclerosis, Coronary Restenosis, etc. One study explored the effect of obesogenic high-fat diets in experimental animal models and found the association of increased DNA methylation in the promoters of Leptin and PPARG2 genes[36].

Not only this but an increase in DNA methylation was found to be associated with the overfeeding of high-fat diets (both polyunsaturated and saturated fat) in adipose tissue, lipid metabolism, and adipose tissue pathways [37]. Although only a very small fraction of studies have been conducted in the case of VTE & DNA methylation, DNA methylation's increasing epigenetic dominance in the cardiovascular scenario is a clear indication of its probable link to the pathophysiology of VTE[38-45].

Lastly, the protein-protein interaction network revealed the L-2-hydroxy glutaric aciduria pathway to be highly enriched in both hypomethylated gene sets as well as in hypermethylated gene sets. The clinical manifestations of this disease represent slow progressive intellectual deterioration, motor nerve impairment, spasticity, dystonia followed by mental retardation and hypotonia[45-47]. Although not much information has been found regarding L2HGDH gene muscle and its methylation status in any cardiovascular diseases, the involvement of the citric acid cycle and pyruvate metabolism (Genecards) makes it a vulnerable target to be explored from the metabolic perspective of cardiovascular diseases[48-50]. Hence, there is a potential for the L2HGDH gene to be associated with any clinical condition concerning CVDs, and the involvement of the Citric acid cycle and Pyruvate metabolism may open a door of new possibilities in VTE initiation.

5. CONCLUSION

Epigenetic and post-translational alterations that control mtDNA and mitochondrial proteins enable communication between the nucleus and the mitochondria which in turn helps to maintain cellular health and balance. Alterations in metabolism, circadian rhythm disturbances, and cancer can all be caused by mitochondrial malfunction, which manifests as changed gene expression and ATP generation as a result of epigenetic modifications. To accomplish this, mitochondrial DNA must reliably be copied, transcribed, translated, and replicated despite the ongoing assault from endogenous and environmental agents. This study aimed to find out the methylation pattern in both high-altitude and sea-level VTE patients. The gene enrichment and ontology studies established several pathway links which are comprised of both nuclear and mitochondrial genes. Post-enrichment assessment of the hypermethylated and hypomethylated genes, citric acid cycle, and NADH dehydrogenase pathways were repeatedly highlighted in KEGG pathway results. Biological processes as well as molecular function highlighted the same mentions. Past experimental pieces of evidence have highlighted the mutual involvement of mitochondrial and nuclear genes in the pathophysiology of several cardiovascular diseases through the epigenetic modification of DNA and histone methylation. Imbalance in the substrate oxidation (NADH/FADH2) of the TCA cycle by the Oxidative phosphorylation pathway in mitochondria could be seen causing a lot of pathological conditions that later become the reason for several cardiovascular problems.

Similarly, NADH dehydrogenase subunit genes have also been found to contribute to the causation of stroke. All this evidence suggests the exploration of nuclear DNA methylation along with mitochondrial DNA methylation to understand several nuances related to CVDs. This approach could also lead to the discovery of mitochondrial DNA methylation's contribution and its involvement in the pathophysiology of Venous thromboembolism, as already nuclear DNA methylation's role has been established in this regard. As a strong and reliable technique like other clinical parameters, DNA methylation could be used as a biomarker discovery and early diagnosis of VTE in the human population.

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