Epigenetics and Familial Hypercholesterolemia: a methylation study on the three canonical genes

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Abstract

Familial hypercholesterolemia (FH) is characterized by high low-density lipoprotein cholesterol (LDL-C) levels and a high risk of early coronary heart disease. Structural alterations in the LDLR, APOB, and PCSK9 genes were not found in 20–40% of patients diagnosed using the Dutch Lipid Clinic Network (DCLN) criteria. We hypothesized that methylation in canonical genes could explain the origin of the phenotype in these patients. This study included 62 DNA samples from patients with a clinical diagnosis of FH according to the DCLN criteria, who previously tested negative for structural alterations in the canonical genes, and 47 DNA samples from patients with normal blood lipids (control group). All DNA samples were tested for methylation in the CpG islands of the three genes. The prevalence of FH relative to each gene was determined in both groups and the respective prevalence ratios (PRs) were calculated. The methylation analysis of APOB and PCSK9 was negative in both groups, showing no relationship between methylation in these genes and the FH phenotype. As the LDLR gene has two CpG islands, we analyzed each island separately. The analysis of LDLR-island1 showed PR = 0.982 (CI 0.33–2.95), also suggesting no relationship between methylation and the FH phenotype. Analysis of LDLR-island2 showed a PR of 4.12 (CI 1.43–11.88), indicating a possible association between methylation on this island and the FH phenotype.

Introduction

Familial hypercholesterolemia (FH) affects one in 310 individuals in the general population and is characterized by defects in low-density lipoprotein (LDL) catabolism, resulting in very high levels of LDL cholesterol (LDL-C). FH is associated with the early onset of atherosclerotic cardiovascular disease, and its origin is related to structural alterations in three canonical genes (CG), LDLR, APOB, and PCSK9, which encode proteins with direct functions in LDL catabolism. \([1, 2]\]

The clinical diagnosis of FH is usually based on the Dutch Lipid Clinic Network (DCLN) criteria. This method involves a list of criteria leading to a score that eventually defines a diagnosis as “possible” (score 3 to 5), “probable” (score 6 to 8), or “definitive” (score > 8). \([2]\]

In 60–80% of cases, it is possible to detect variants (changes in sequencing, deletion, or duplication) in at least one CG. However, the search for genetic abnormalities is inconclusive in 20–40% of patients with the FH phenotype. \([3]\] One possible explanation is polygenic heritage. \([4]\] Extensive genome-wide association studies (GWASs) of LDL allows the construction of polygenic risk scores (PRSs) for LDL-C in genotyped subjects.\([5]\] Multiple reports have indicated that 20–30% of patients who have clinical FH have a high LDL PRS that may provide the basis of their polygenic hypercholesterolemia.\([6]\]

Another theory is that epigenetic mechanisms such as DNA methylation silence at least one CG, despite its structural integrity. In humans, this phenomenon is observed in specific regions of DNA called CpG islands, where sequences of cytosine-guanine dinucleotides occur. Once methylated, gene expression is silenced.\([7]\]

The LDLR gene, the most common CG related to the phenotypic expression of FH, has two CpG islands in the promoter region, which may predispose patients to undergo methylation. APOB and PCSK9 have one CpG island in their respective promoter regions.\([8]\]

Methylation as a cause of the FH phenotype has been investigated by comparing mutation-positive and mutation-negative FH groups, revealing significantly hypomethylated CpG sites in CPT1A. No differences were observed in the other genes.\([9]\]
The influence of the FH phenotype in pregnant women on offspring has also been considered. There may be an increased risk among babies exposed to FH during pregnancy, when epigenetic events occur most frequently.[10]

Our hypothesis is that there may be an association between the methylation status of at least one structurally intact CG and the FH phenotype. The aim of this study was to test this association.

**Methods**

This study investigated the association between the DNA methylation status in structurally intact CGs and patients clinically diagnosed with FH according to the DCLN criteria.

Convenience samples were used for both groups. The inclusion criteria for the study and control groups were the presence and absence, respectively, of a clinical diagnosis of FH according to DCLN scores.[11]

The study group included 62 DNA samples from individuals with DCLN scores over 5 and therefore diagnosed as FH “probable” or “definitive.” These samples were obtained from the Hipercol Brasil Project team biorepository. All these samples had previously tested negative for any structural alterations.[12]

The control group consisted of 47 DNA samples from the Ribeirão Preto School of Medicine biorepository from individuals with at least two previous normal blood LDL-C and triglyceride (TG) levels. We considered this a sufficient condition to assume a negative FH diagnosis. Normal blood levels were LDL-C < 100 mg/dL and TG < 100 mg/dL for individuals between 2 and 20 years of age and LDL-C < 130 mg/dL and TG < 150 mg/dL for individuals over 20 years of age. The mean difference in LDL-C levels between the groups was significant (p < 0.0001). Table 1 presents the details of these populations.
Table 1

Clinical characteristics of the study (FH+) and control (FH-) groups.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>FH+ Group (n = 62)</th>
<th>FH− Group (n = 47)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td>Male</td>
<td>Female</td>
</tr>
<tr>
<td></td>
<td>30 (48.4%)</td>
<td>27 (42.6%)</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td></td>
</tr>
<tr>
<td></td>
<td>32 (51.6%)</td>
<td>27 (57.4%)</td>
</tr>
<tr>
<td>Age, years</td>
<td>2–20</td>
<td>37 (78.7%)</td>
</tr>
<tr>
<td></td>
<td>21–40</td>
<td>10 (21.3%)</td>
</tr>
<tr>
<td></td>
<td>41–60</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>&gt; 60</td>
<td>-</td>
</tr>
<tr>
<td>Ethnicity</td>
<td>White</td>
<td></td>
</tr>
<tr>
<td></td>
<td>36 (58.1%)</td>
<td>38 (80.9%)</td>
</tr>
<tr>
<td></td>
<td>Black</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>4 (6.5%)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Brown</td>
<td>2 (3.2%)</td>
</tr>
<tr>
<td></td>
<td>9 (19.1%)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>No ID</td>
<td>20 (32.2%)</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>&lt; 20</td>
<td>NAD</td>
</tr>
<tr>
<td></td>
<td>20–25 (normal)</td>
<td>NAD</td>
</tr>
<tr>
<td></td>
<td>25–30 (overweight)</td>
<td>NAD</td>
</tr>
<tr>
<td></td>
<td>30–35 (class 1 obesity)</td>
<td>NAD</td>
</tr>
<tr>
<td></td>
<td>35–40 (class 2 obesity)</td>
<td>NAD</td>
</tr>
<tr>
<td></td>
<td>&gt; 40 (class 3 obesity)</td>
<td>NAD</td>
</tr>
<tr>
<td>LDL-cholesterol</td>
<td>&lt; 100 mg/dL</td>
<td>37 (78.7%)</td>
</tr>
<tr>
<td></td>
<td>&lt; 130 mg/dL</td>
<td>10 (21.3%)</td>
</tr>
<tr>
<td></td>
<td>130–189 mg/dL</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>190–249 mg/dL</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>250–329 mg/dL</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>≥ 330 mg/dL</td>
<td>-</td>
</tr>
<tr>
<td>Family history of FH</td>
<td>56 (90.3%)</td>
<td>NA</td>
</tr>
<tr>
<td>Clinical history of FH</td>
<td>32 (51.6%)</td>
<td>NA</td>
</tr>
<tr>
<td>Changes in physical examination</td>
<td>4 (6.4%)</td>
<td>NA</td>
</tr>
</tbody>
</table>

All samples from both groups were subjected to modification of genomic DNA with sodium bisulfite.[13] Methylation analysis was conducted using a methylation-sensitive high-resolution melting assay (MeltDoctor HRM Master Mix kit; ThermoFisher®) and polymerase chain reaction (PCR) (7500 Fast Real-Time PCR System; ThermoFisher®). The PCR protocol was to raise the temperature to 95°C for 10 minutes, followed by 40 cycles of 1) denaturation at 95°C for 15 seconds, 2) annealing at 58°C for 1 minute, and 3) extension at 72°C for 1 minute.
Melting curves were established using the melting temperature (MT) of each sample, with an approximation level of 0.1ºC. MT refers to the system temperature at which 50% of DNA is double-stranded and the other 50% is single-stranded open.[14] The MT of each sample was analyzed using comparisons of 0% and 100% methylated standard MT as parameters.[15]

As the MT is directly related to the methylation status of the sample, it is possible to estimate the percentage of methylated samples by comparing the relative position of the samples’ MT between the respective 0% and 100% standards’ MT.[15]

From MTs of 0% and 100% methylated standards, the methylation percentages of each sample were calculated by proportionality rule[16], according to the following equation (MT% = sample's methylation percentage; MTS = sample's MT; MT0% = 0% methylated standard’s MT; MT100% = 100% methylated standard’s MT):

\[
MT\% = \left[MTS - MT0\%\right] \div \left(MT100\% - MT0\%\right)
\]

We considered samples with MT% ≥90% to be methylated. This pattern was considered a positive exposition for the FH phenotype to run the prevalence calculations. Samples with MT% <0% or >120% were considered anomalies and excluded from the final analysis.

To calculate prevalence, we considered the methylation status of each CpG island separately. Therefore, we determined the prevalence of the FH phenotype and methylation status in four different ways: APOB, PCSK9, LDLR-island1, and LDLR-island2.

Finally, the prevalence ratio (PR) and 95% confidence interval (CI) were calculated for each CpG island.[17]

This study was submitted and approved by the Ribeirão Preto Clinics Hospital of São Paulo University’s Ethics Committee (Comitê de Ética em Pesquisa do Hospital das Clínicas de Ribeirão Preto da Universidade de São Paulo). Informed consent was waived with accordance of the Ribeirão Preto Clinics Hospital of São Paulo University’s Ethics Committee (Comitê de Ética em Pesquisa do Hospital das Clínicas de Ribeirão Preto da Universidade de São Paulo). Original document is available in Supplementary Information.

All methods were performed in accordance with the relevant guidelines and regulations.[18]

Results

• Study group

Our team received 218 DNA samples from the Hipercol Brasil biorepository. Of these, 156 did not meet the inclusion criteria; 87 DNA samples were from patients who had a DCLN score < 6, 82 had an insufficient amount of genetic material to run the tests, 16 had mutation events, 15 had not been tested for mutation events, 16 were from patients who did not have all the required clinical data, and two were from patients with blood TSH levels > 5.0 mU/L. The 62 remaining DNA samples formed the study group.

All samples were subjected to methylation analysis of the CpG islands of the APOB and LDLR-island1 genes. Subsequently, one sample was excluded because of insufficient genetic material. The 61 samples remaining were submitted to methylation analysis of the CpG islands PCSK9 and LDLR-island2.
After methylation tests, some DNA samples were excluded from the final analysis: 49 exclusions of \textit{LDLR}\textsubscript{island1} analysis, of which 42 were for anomalous melting curve results and 7 for MT\% <0\% or >120\%; 13 exclusions of \textit{APOB} analysis for MT\% <0\% or >120\%; and 11 exclusions of \textit{LDLR}\textsubscript{island2} analysis for MT\% <0\% or >120\%.

The final study group consisted of 13 samples for \textit{LDLR}\textsubscript{island1}, 49 samples for \textit{APOB}, 50 samples for \textit{LDLR}\textsubscript{island2}, and 61 samples for \textit{PCSK9}. Figure 1 shows the process for selecting the study group DNA samples.

**Control group**

We had 60 DNA samples from patients who had participated in previous clinical trials and were available from the Ribeirão Preto School of Medicine’s biorepository. Of these, 13 were excluded, of which 10 were from patients with blood TG > 150 mg/dL and three from patients with insufficient genetic material. The remaining 47 DNA samples formed the control group and were subjected to methylation analysis of the four CpG islands.

After the methylation tests, 13 DNA samples were excluded for \textit{LDLR}\textsubscript{island1}, one for \textit{APOB}, 21 for \textit{LDLR}\textsubscript{island2}, and two for \textit{PCSK9}. All exclusions were due to MT\% <0\% or >120\%. The final control group consisted of 34 samples for \textit{LDLR}\textsubscript{island1}, 46 samples for \textit{APOB}, 26 samples for \textit{LDLR}\textsubscript{island2}, and 45 samples for \textit{PCSK9}. Figure 2 shows process for selecting the control group DNA samples.

**Analysis of methylation**

For \textit{LDLR}\textsubscript{island1}, 13 FH\+ and 34 FH\- samples were eligible for determining prevalence. In the study group, three samples were considered methylated, and 10 samples were not methylated. In the control group, eight samples were methylated, and 26 samples were not methylated. The prevalence of disease was 27.3\% among those exposed and 27.8\% among those not exposed (PR = 0.98; CI = 0.33–2.95).

For \textit{APOB}, 49 FH\+ and 26 FH-samples were eligible for prevalence determination. None of the samples from either group were methylated.

For \textit{LDLR}\textsubscript{island2}, 50 FH\+ samples and 46 FH\- samples were eligible for determining prevalence. In the study group, 47 samples were considered methylated, and three samples were not methylated. In the control group, 29 samples were methylated and 17 were not methylated. The prevalence of disease 61.8\% among those exposed and 15.0\% among those not exposed (PR = 4.12; CI = 1.43–11.88).

For \textit{PCSK9}, 61 FH\+ and 45 FH\- samples were eligible for determining prevalence. None of the samples from either group were methylated.

Table 2 shows the contingency tables.
Table 2
- Comparisons of methylation patterns and clinical diagnosis for each island analysis

<table>
<thead>
<tr>
<th>LDLR/Island-1</th>
<th>FH+</th>
<th>FH-</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methylated</td>
<td>3 (27.3%)</td>
<td>8 (72.7%)</td>
<td>11</td>
</tr>
<tr>
<td>Not methylated</td>
<td>10 (27.8%)</td>
<td>26 (72.2%)</td>
<td>36</td>
</tr>
<tr>
<td>Total</td>
<td>13</td>
<td>34</td>
<td>47</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>LDLR/Island-2</th>
<th>FH+</th>
<th>FH-</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methylated</td>
<td>47 (61.8%)</td>
<td>29 (38.2%)</td>
<td>76</td>
</tr>
<tr>
<td>Not methylated</td>
<td>3 (15.0%)</td>
<td>17 (85.0%)</td>
<td>20</td>
</tr>
<tr>
<td>Total</td>
<td>50</td>
<td>46</td>
<td>96</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>APOB</th>
<th>FH+</th>
<th>FH-</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methylated</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Not methylated</td>
<td>49 (65.3%)</td>
<td>26 (34.7%)</td>
<td>75</td>
</tr>
<tr>
<td>Total</td>
<td>49</td>
<td>26</td>
<td>75</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>PCSK9</th>
<th>FH+</th>
<th>FH-</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methylated</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Not methylated</td>
<td>61 (57.5%)</td>
<td>45 (42.5%)</td>
<td>106</td>
</tr>
<tr>
<td>Total</td>
<td>61</td>
<td>45</td>
<td>106</td>
</tr>
</tbody>
</table>

**Discussion**

FH is a common disease associated with early onset of coronary artery disease. The genetic etiology of FH remains unknown in 20–40% of patients with a clinical diagnosis. We postulate that epigenetics might explain some of these cases. DNA methylation in the gene promoter region prevents the opening of the double strand during transcription, resulting in non-expression (or silencing) of this gene.

Among the four CpG islands studied, the methylation pattern of one (LDLR-island2) differed between the groups; therefore, the study group (FH+) had a higher proportion of methylation genes, which may demonstrate an association between methylation on this island and the FH phenotype.

A PR of 4.12 indicates that individuals with a methylation pattern higher than 90% had four times the risk of FH when compared to individuals with lower methylation patterns. Silencing of this gene could be a mechanism underlying the FH phenotype.

Differences in the methylation of the other genes were not statistically significant. However, the finding of methylation in specific CpG islands of LDLR, which is related to most molecular etiologies of FH, could explain the FH phenotype.
The use of convenience samples has some limitations. The two groups were not homogeneous because the samples originated from different populations. However, the inclusion criterion established for each group was the presence or absence of a clinical diagnosis of FH. Individuals from the control group had at least two normal LDL-C blood levels, which guaranteed that they did not have FH. Working with homogeneous groups and larger samples, it may be possible to observe better results for \textit{LDLR}-island, \textit{APOB}, and \textit{PCSK9} CpG islands.

Another limitation is that high LDL-C polygenic scores, which may explain up to 20\% of the causes of the FH phenotype, were not calculated.[3, 19] However, to the best of our knowledge, this is the first study specifically designed to test the association between epigenetic alterations and the FH phenotype.

We found an association between DNA methylation and FH phenotypic expression. Despite the limitations already described, we tested the four CpG islands on the CGs, which opens the possibility of explaining the origin of the FH phenotype in patients in whom variants in the CGs were not encountered. Epigenetic mechanisms, particularly DNA methylation, are already well established in the literature.

More studies with larger sample sizes are necessary to better understand the pathogenic mechanisms of FH.

\textbf{Declarations}

\textbf{Additional information}

RDS received a scholarship from Conselho Nacional de Pesquisa e Desenvolvimento Tecnológico, Brazil (CNPq) #303734/2018-3. Funding from Sociedade Hospital Samaritano and Ministério da Saúde (PROADI-SUS; SIPAR:25000.180.672/2011-81) and FAPESP (grant 2013/17368-0) is gratefully acknowledged for the Hipercol Brasil program.

This study was made possible by the financial incentives of FAPESP, an entity that RAZ and RDRJ gratefully acknowledge.

\textbf{Conflicts of interest statement}

RDS received honoraria related to consulting, research, and/or speaker activities from Abbott, Ache, Amgen, Astra Zeneca, Esperion, EMS, Getz Pharma, Kowa, Libbs, Novo-Nordisk, Novartis, Merck, MSD, Pfizer, PTC Therapeutics, and Sanofi/Regeneron.

RAZ and RDRLJ have no conflicts to state.

\textbf{Data availability statement}

All data generated or analyzed during this study are included in this published article (and its Supplementary Information files).

\textbf{Authors contribution statement}

Zorzo RA was the main author. He was responsible for the conception and design, coordinated all the research paths, run the statistical analysis, and wrote the hole article.
Liberatore-Jr RDR was Zorzo's mentor on PhD project that rose the project. Helped and guided Zorzo through all paths since the conception until submission of the research.

Suen VMM, Santos JE, Liberatore-Jr RDR and Honorato ALSC made up the team for interpretation and analysis of data and contributed to acquisition of equipment for methylation tests.

Silva-Jr WA helped to study design particularly on genetic issues of LDLR, APOB and PCSK9 genes.

Suazo VK executed all methylation tests of all samples.

Jannes CE, Pereira A, and Krieger JE worked on the mutation analysis of all FH+ Group samples prior to the methylation analysis.

Santos RD was responsible on the institution that provided FH+ group samples and was the leader of the team composed by Jannes, Pereira and Krieger.

References


**Figures**

![Diagram of sample inclusion and exclusion criteria](image-url)
Figure 1

Flowchart of sample inclusion for the FH+ group

Samples available for the control group (60)

Not included (13)
• TG > 150 mg/dL (10)
• Insufficient amount of genetic material (3)

Included samples (47)

LDLR/Island-1 Methylation analysis (47)

Exclusions (13)
• Melting temperature < 0% or > 120% (13)

Samples submitted to methylation analysis (34)

APOB Methylation analysis (47)

Exclusions (1)
• Melting temperature < 0% or > 120% (1)

Samples submitted to methylation analysis (46)

LDLR/Island-2 Methylation analysis (47)

Exclusions (21)
• Melting temperature < 0% or > 120% (21)

Samples submitted to methylation analysis (26)

PCSK9 Methylation analysis (47)

Exclusions (2)
• Melting temperature < 0% or > 120% (2)

Samples submitted to methylation analysis (45)

Figure 2

Flowchart of sample inclusion for the FH- group

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementaryinformation1MeltingresultsfromLDLRIsland1FHGroup.docx
- Supplementaryinformation2MeltingresultsfromLDLRIsland1FHGroup.docx
- Supplementaryinformation3MeltingresultsfromLDLRIsland2FHGroup.docx
- Supplementaryinformation4MeltingresultsfromLDLRIsland2FHGroup.docx
- Supplementaryinformation5Contingencytables.docx
- Supplementaryinformation6ApprobationfromEthicsCommitteeandaccordancetowaveofInformedconsent.docx