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Familial lecithin-cholesterol acyltransferase deficiency: If so rare, why so frequent in the state of Piauí, northeastern Brazil?

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ABSTRACT

Lecithin-cholesterol acyltransferase (LCAT), an enzyme that participates in lipoprotein metabolism, plays an important role in cholesterol homeostasis. Mutations in the *LCAT* gene can cause two rare genetic disorders: familial LCAT deficiency (FLD), which is characterized by corneal opacities, normocytic anemia, dyslipidemia, and proteinuria progressing to chronic renal failure, and fish-eye disease (FED), which causes dyslipidemia and progressive corneal opacities. Herein, we report six suspected cases of FLD in the backlands of Piauí, located in northeast Brazil. A genetic diagnosis was performed in index cases. Among these, a further investigation was performed to identify new cases in the families. In addition, molecular analyses were performed to verify the levels of consanguinity within families and the existence of a genetic relationship between them. All six index cases were confirmed as FLD with an identical mutation (c.803G > A, p.R268H). The genetic investigation confirmed another 7 new cases of FLD, 52 heterozygous and 6 individuals without mutations. The rate of consanguinity revealed that marriages within the family did not contribute to the high number of FLD cases within the restricted region. The elders of each family (patriarchs and matriarchs) were subjected to a kinship analysis and were more genetically related to each other than the control group. Bayesian analysis was implemented to confirm the hypothesis of connectivity among patriarchs and matriarchs and indicated that they were genetically more related to each other than would be randomly expected, thus suggesting the occurrence of a possible founder effect in these families.

1. Introduction

In medicine, the accuracy of diagnosis depends on the physician's observational capacity, complemented by clinical and laboratory tests. The initial observation of bilateral corneal opacity in a young woman with nephrotic proteinuria and dyslipidemia, along with low high-

Abbreviations: FED, Fish-eye disease; FLD, familial lecithin-cholesterol acyltransferase deficiency; HDL, high-density lipoprotein; LCAT, lecithin-cholesterol acyltransferase.

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density lipoprotein (HDL) levels, confirmed by molecular diagnosis, verified the diagnosis of the first case of lecithin-cholesterol acyltransferase (LCAT) deficiency in the state of Piauí, Brazil (unpublished data).

The *LCAT* gene (~4.5 kb) is located on the long arm of chromosome 16 (region 16q22) and contains 6 exons encoding a 416 amino acid glycoprotein, approximately 63 kDa in molecular mass [1,2]. LCAT mediates the synthesis of cholesterol esters in plasma, which are incorporated into the interior of HDL, thus facilitating the formation and maturation of circulating HDL [1,3]. Then, the mature spherical HDL delivers cholesterol to the liver, where it undergoes degradation and elimination [4–7].

Notably, mutations in the *LCAT* gene result in deficient enzyme activity which causes drastic alterations in the lipoprotein profile, showing an increased percentage of unesterified cholesterol and low levels of HDL-c. Furthermore, alterations in lipoprotein distribution are also observed, such as loss of mature spherical HDL, increase in discoid nascent HDL particles, increased levels of triglycerides and low levels of LDL-c [8–10].

LCAT deficiency was first described by Norum and Gjone in 1967 in Norway, and to date, 117 mutations have been identified along the *LCAT* gene [11,12]. Among genetic defects described, the majority (77 mutations) resulted in the familial LCAT deficiency phenotype (FLD; OMIM# 245900), 12 in the fish-eye disease phenotype (FED; OMIM# 136120), and 28 remain to be classified [12].

FLD is a severe form with absent or complete inactivity of the enzyme, resulting in dyslipidemia, corneal opacities, anemia, and progressive nephropathy. FED is a milder phenotype that causes dyslipidemia and progressive corneal opacities [13]. Approximately 130 cases of partial or complete LCAT deficiency [12] have been reported in different countries worldwide, highlighting intense and gradual population migration. Norum et al. show information of 38 FLD patients from seven different families living in Northern Brazil [14].

Recently, a case of FLD was identified at a nephrology department in the state of Piauí, northeastern Brazil. This discovery led to a series of investigations that identified five additional cases carrying the same pathology in different families in cities within the same state. These had the same clinical characteristics as the first case, with variations in the glomerular filtration rate from normal to stage 5. Therefore, to understand the high frequency of this autosomal recessive disease within a restricted region, the present study was undertaken to identify mutations present in affected families, search for new cases within families, measure levels of consanguinity, and determine whether a genetic relationship exists between families diagnosed with FLD.

2. Materials and methods

2.1. Patients and control population

Blood samples from six probands and their relatives ($n = 71$) were collected for genetic diagnosis. For molecular tests and genotypic comparisons between individuals of the same family and within families, DNA of 56 individuals belonging to five families (F01 = 6, F02 = 11, F03 = 13, F04 = 23, and F05 = 3) and 36 negative controls (noncarriers of the disease residing in the state of Piauí) was extracted. Other family members did not participate in the genotypic analysis, given the lack of biological material.

The experiments were approved by the Human Research Ethics Committee of the Federal University of Piauí, under protocol number 3,662,537/2019. In addition, all participants provided written informed consent.

2.2. Genetic diagnosis and molecular analysis

For genetic diagnosis, the *LCAT* gene (NM_000229.1) was amplified by PCR. DNA sequencing was performed on both strands of the entire coding region and the highly conserved exon-intron splice junctions.

After diagnosis, the levels of consanguinity and genetic relationships among each proband family were investigated using molecular analysis. Genomic DNA was extracted from peripheral blood using the Wizard Genomic DNA Purification Kit (Promega, WI, USA), according to the manufacturer's instructions. DNA samples were normalized to 2 ng/μL. Samples from family members and negative controls were genotyped using the VeriFiler™ Express PCR Amplification Kit (Thermo Fischer, MA, USA), which amplified 23 autosomal microsatellite loci (D3S1358, vWA, D16S539, CSF1PO, TPOX, D8S1179, D21S11, D18S51, D2S441, D19S433, TH01, FGA, D22S1045, D5S818, D13S317, D7S820, D10S1248, D1S1656, D12S391, D2S1338, D6S1043, Penta D, Penta E), a polymorphic indel located on the Y chromosome, and the *amelogenin* gene, located on the X chromosome.

PCR was performed with a GeneAmp PCR 9700 Thermocycler (Life Technologies, CA, USA) using 50 ng of genomic DNA. After amplifying the described loci, genotypes were determined by capillary electrophoresis in an ABI PRISM 3500xl Genetic Analyzer (Applied Biosystems, MA, USA), following the manufacturer's instructions. GeneMapper *ID-X* software v1.4 (Applied Biosystems, MA, USA) was used to perform the analysis.

2.3. Measurement of intrafamilial inbreeding levels

The inbreeding coefficients of families were calculated to assess the level of consanguinity within each family. Analyses were performed based on Wright's F_{IS} index [15] estimated in the GenAlEx program [16] as follows:

$$F_{IS} = (H_S - H_I)/H_S$$

where F represents the mean level of consanguinity of individual I within group S, considering the expected heterozygosity within each group (H_S) in relation to the averages observed per individual (H_I).

The F index ranges from −1 to +1, with positive values close to +1 representing high inbreeding rates at the local level, values close to zero representing random marriages, and negative values close to −1 representing non-random marriages, preferred among unrelated individuals.

2.4. Kinship analysis

The GenAlEx program was also used to calculate the genetic relatedness matrix between each evaluated individual. Accordingly, a likelihood test based on the Queller and Goodnight r index was used [17]. The estimator was initially developed to estimate similarities between populations but was adapted to obtain relatedness rates between individuals [18–20]. Thus, the relationship between an individual x and an individual y at *locus* l is:

$$\widehat{r}_{xy,l} = [0.5(I_{ac} + I_{ad} + I_{bc} + I_{bd}) - p_a - p_b]/[1 + I_{ab} - p_a - p_b]$$

where $I_{ac} = 1$ when the a allele of individual x is identical to the c allele of individual y, or $I_{ac} = 0$ when the alleles are not the same, and so on; p_a and p_b represent the frequency of the a and b alleles at locus l; $I_{ab} = 1$ when a and b alleles of individual x are identical, or $I_{ab} = 0$ if they are not identical.

Considering L loci analyzed, the relationship between two individuals will be represented as:

$$\widehat{r}_{xy} = \left(\sum_{l=1}^{L} \widehat{r}_{xy,l} + \widehat{r}_{yx,l}\right) \Big/ 2L$$

The estimate of pairwise relatedness was calculated for all participants. Subsequently, the mean intrafamilial kinship and mean kinship of the control group were evaluated. Additionally, the average kinship was calculated by considering only elders (matriarchs and patriarchs) of

each family. This last evaluation was performed to investigate possible family links between these ancestral individuals, suggesting the possibility of a unique origin for the *LCAT* gene mutation identified in this population.

2.5. Genetic cluster analysis among the elders

Next, considering the possibility of a link between evaluated families, genetic data of elders (*i.e.*, patriarchs and matriarchs) of each family were isolated from the other family members ($N = 10$) and submitted to a cluster test along with the control group. The analysis was performed using the Structure 2.3.3 program [21], which implements a Bayesian clustering algorithm, where *a priori* information regarding the origin of samples was not used. Analyses were carried out according to the admixture model, considering no linkage disequilibrium between markers. Overall, 10 runs with a burn-in of 100,000 and a run length of 400,000 iterations (Markov and Monte Carlo chains) were performed for several possible clusters varying from K = 1 to K = 7. The maximum number of tested groups (K = 7) was defined in a semi-arbitrary manner, considering the presence of five families tested with DNA information and the possibility of evaluating potential partitions among samples given their genetic information. Thus, considering that the ancestors belonged to the same genetic group, they are expected to remain grouped (without partition) among them, while separate from the negative control group. Conversely, if there is separation (partition) between the ancestor members, it is suggested that they have different genetic origins.

The analytical results were presented considering the average of 10 replicates performed in the analyses in a bar graph considering the inferred ancestry of each individual. The graph was obtained using the *pophelper* package [22] available for the R computing environment [23].

3. Results

Herein, six apparently unrelated female patients carrying the mutation (c.803G > A p.R268H) in exon 6 of the *LCAT* gene were diagnosed with FLD. With the molecular confirmation of homozygosity in the index cases, the respective families underwent a familial investigation, resulting in another 7 diagnoses of FLD, 52 heterozygous and 6 individuals without mutations, as shown in Fig. 1.

3.1. Intrafamily inbreeding levels

Next, inbreeding levels were measured due to the identification of several cases, including cases within the same family. Accordingly, samples of 56 family members were genotyped by the amplification of autosomal microsatellite loci, a polymorphic indel located on the Y chromosome, and the *amelogenin* gene, located on the X chromosome.

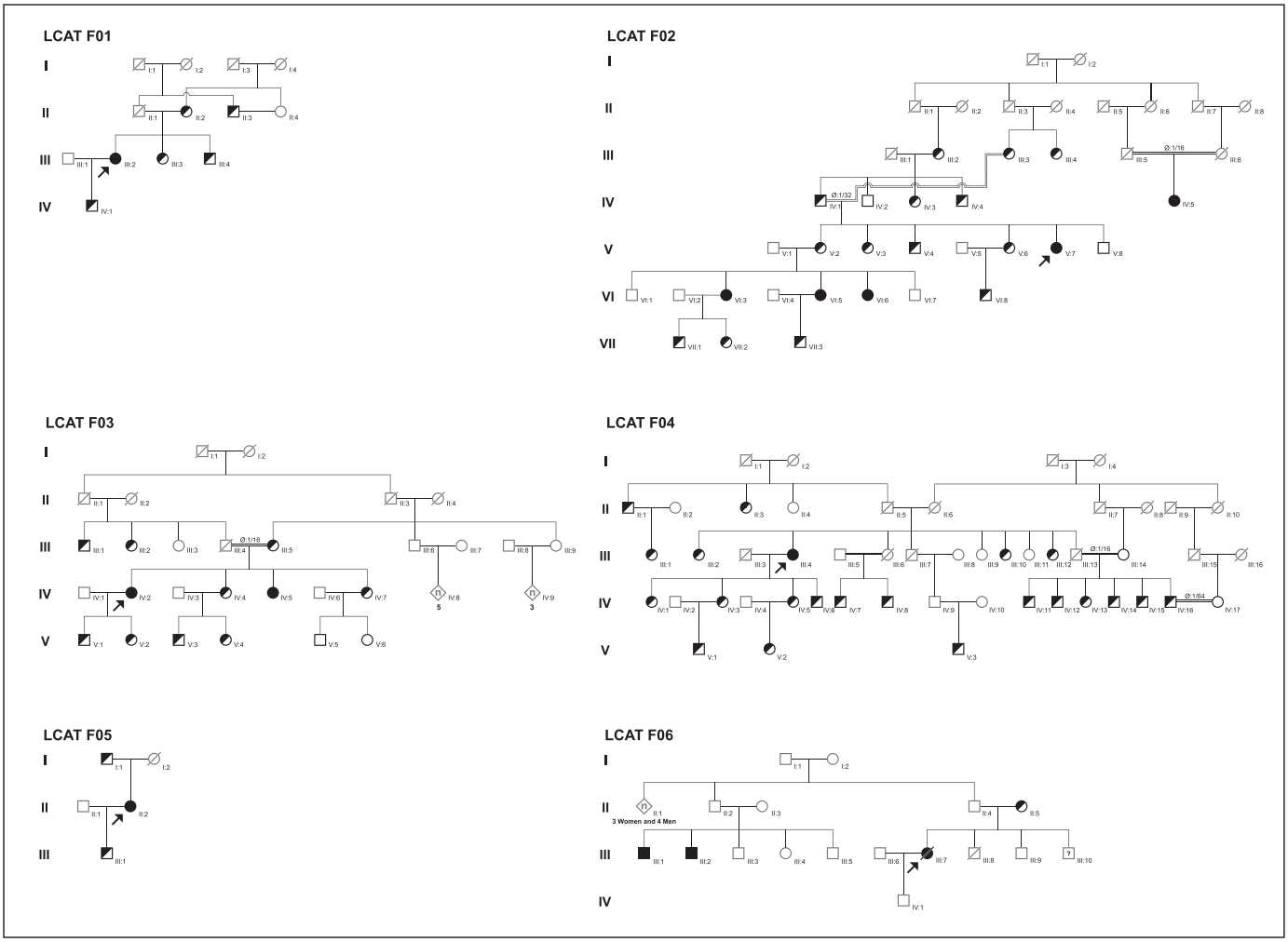

Fig. 1. Pedigree of six families with a molecular diagnosis of familial LCAT deficiency. Squares indicate males; circles indicate females; diamond indicates unspecified sex; filled symbols indicate homozygote carriers; half-filled symbols indicate heterozygote carriers; white symbols indicate noncarrier individuals; slashed symbols indicate deceased individuals; squares and circles with gray borders indicate individuals not available for analysis. LCAT, lecithin-cholesterol acyltransferase.

Finally, inbreeding coefficients were calculated using genotypic frequency. As shown in Table 1, there was no evidence of intrafamilial consanguinity, and the F values were all negative, ranging from −0.442 to −0.045.

3.2. Kinship levels between individuals of the same family, between the ancestor members and negative controls

We next attempted to identify the source underlying the numerous cases of FLD within the same region. Accordingly, a kinship analysis was performed among the members of each family. A further analysis was performed among patriarchs and matriarchs (F01 to F05, n = 10) of each family to detect any possible link between families (Fig. 2). Based on the intrafamilial kinship analysis, the r index ranged between 0.17 and 0.39, and the negative control approximated zero. On grouping the elders of each family, the r index was 0.07.

3.3. Genetic cluster analysis between elders of each family and negative controls

A Bayesian clustering approach, using Structure software, was applied to samples with genotype information (based on microsatellite markers) to determine any trace of genetic connectivity among families. This analysis considered only patriarchs and matriarchs of each family to eliminate strong genetic signals typically observed among close relatives. Then, the genetic information of the examined elders was pooled with control samples without incorporating any *a priori* information. The analysis revealed the membership probabilities of each individual considering 6 different scenarios, where samples were assigned to 2–7 different genetic groups (K values). The results showed that patriarchs and matriarchs were persistently grouped together, belonging to the same cluster (always with the same color pattern), thus indicating the occurrence of some level of coancestry among them. Furthermore, this result was consistently obtained regardless of the K value (Fig. 3). It is also worth mentioning that the elderly diverged from negative controls in all scenarios of K.

On diving samples into two groups (K = 2), elders were considered to belong to the "dark blue group," with membership probabilities ranging between 85.25% and 95.14%. Conversely, the probabilities of control individuals belonging to the same dark blue group ranged between 1.46% and 6.86%.

For increasing values of K, a greater number of color groups was observed; this conclusion followed the same pattern, with elders of each family always grouped under one main color. For values of K = 4 and K = 5, elders were preferentially grouped into the "red group," while for K = 6 and K = 7, the orange color was the discriminant group for elders (Fig. 3). It can be expected that the higher the value of K, the smaller the membership probability values within each color, as more scenarios will be considered in the tests. For example, on analyzing K = 7, the elders were mainly grouped in the "orange group" (Fig. 3). Within this genetic group, the membership probabilities among the elderly ranged between 48.19% and 55.42%. Despite the decrease in the probability values for elders, the values were considerably higher than those of control samples considering the same genetic group. Accordingly, the membership probabilities of control individuals ranged from 3.22% to 4.79% for the orange group.

Notably, control subjects consisted of randomly sampled individuals. Therefore, it is speculated that they do not present high membership probability values for larger values of K (unless they are true relatives). Considering K = 7, which was the highest value tested, the membership probabilities among control individuals averaged 14.28% (Standard deviation [SD] = 9.41%), implying that they present almost identical probabilities of belonging to any color group. Therefore, there was no specific color with strong probabilities for these samples, and their relatedness was low and inconclusive (Fig. 3). Conversely, even the lowest value of membership probability was 48.19% for the elderly, with an average of 66.31% (SD = 17.79%) on considering all six scenarios of K, thus indicating the genetic association among these individuals.

Table 1
Genetic and inbreeding estimates within the tested groups considering families (F01 to F05) and negative controls (NC).

Groups	N	Na	Ho	He	F
F01	6	3.739	0.775	0.588	−0.300
F02	11	4.391	0.708	0.619	−0.137
F03	13	4.696	0.729	0.610	−0.194
F04	23	5.870	0.803	0.704	−0.136
F05	3	3.043	0.797	0.548	−0.442
NC	36	8.652	0.826	0.792	−0.045

N = sample size; Na = mean number of alleles per genetic marker tested; Ho = average level of observed heterozygosity within the group; He = Average level of expected heterozygosity within the group; F = inbreeding coefficient.

4. Discussion

In the present study, we detailed the discovery and diagnosis of six cases of familial LCAT disease. After diagnosis, the families were subjected to an in-depth investigation to verify if any other family member was affected by this genetic disorder and accordingly perform genetic counseling. Genetic research was essential for discovering new cases in families, increasing the number of cases by 116.67% in relation to index cases, totaling 13 homozygous cases for the disease (11 females and 2 males) and an impressive 52 of 71 cases of heterozygosity. These data reveal the importance of medical staff who would consider the possibility of rare diseases. Moreover, a laboratory capable of performing molecular diagnostics is another key requirement, which is not feasible in most parts of the world.

Herein, some data were noteworthy. First, all families presented the same mutation (c.803G > A, p.R268H) in exon 6 of the *LCAT* gene. This mutation was first described by Calabresi et al. in a family in Italy [9,24] and later found in a man with compound heterozygous in Norway [25]. Another interesting fact is that the diagnosed families were all located in nearby cities, some in neighboring cities or towns within the same city, all located in the south-central region of Piauí; with the radius between extreme cities approximately of 160 km (Fig. 4). Despite being an extremely rare autosomal recessive disease, the phenotypic frequency of patients with FLD was 1 case for approximately 3388 inhabitants in respective residential cities [26].

Based on the history of the disease, which was first related to the European continent [11], we hypothesized that this mutation was introduced to Brazil during its colonization. This idea is further reinforced by understanding the colonization of Piauí. Brazil was initially colonized along its coastline, leaving its inland unexplored. During the colonization of Brazil, which was predominantly Portuguese, there were some periods when the French and Dutch occupied certain regions of Brazil, the first the island of São Luís in the state of Maranhão, and the last, the hereditary captaincy of Pernambuco, two states neighboring Piauí. The interiorization of Brazil and the colonization of Piauí took place in a second period, during the 17th century, when farmers in search of land for pasture and animal husbandry arrived in Piauí. Thus, the occupation of Piauí occurred in a manner contrary to that of other states in the Northeast of Brazil, from the interior to the coast. The first dwellings were in the interior, where two Portuguese families arrived with servants and slaves and created large farms. Whenever settlement took place, landlords went deeper into the interior, searching for new lands and leaving their people on the farms, which later became towns and villages, thus possibly contributing to genetic isolation [27].

It is worth mentioning that several places where the index cases reported in the present study are still villages or small towns, where practically all inhabitants are genetically related. Given the social segregation, marriages of individuals with the same anomaly may occur,

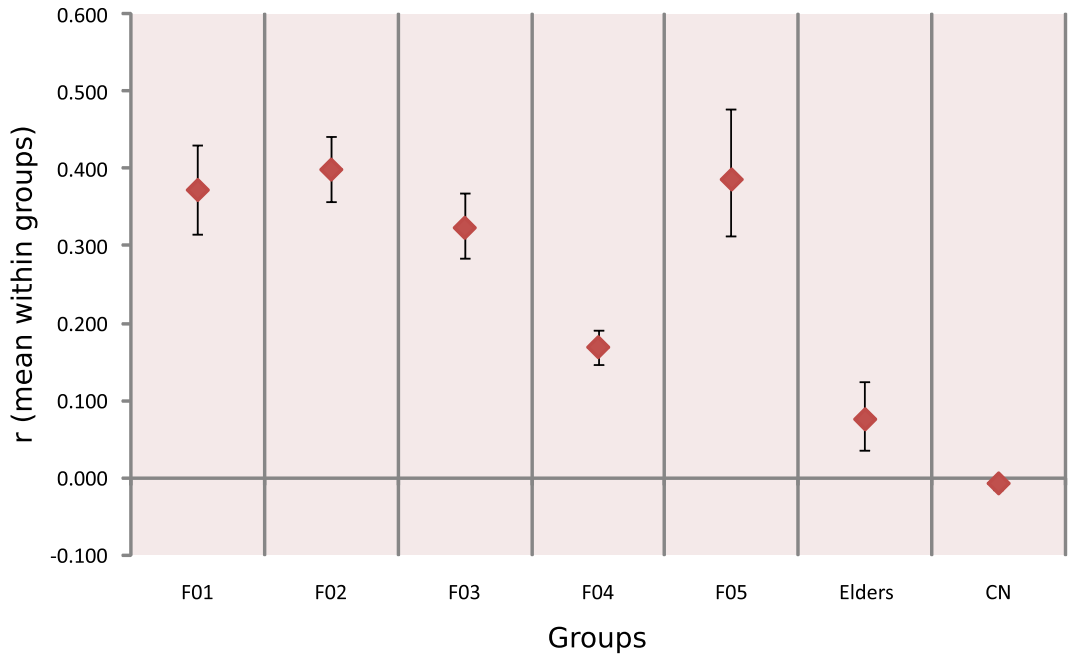

Fig. 2. Medium kinship within families (F01 to F05), between elders of each family and negative controls (NC).

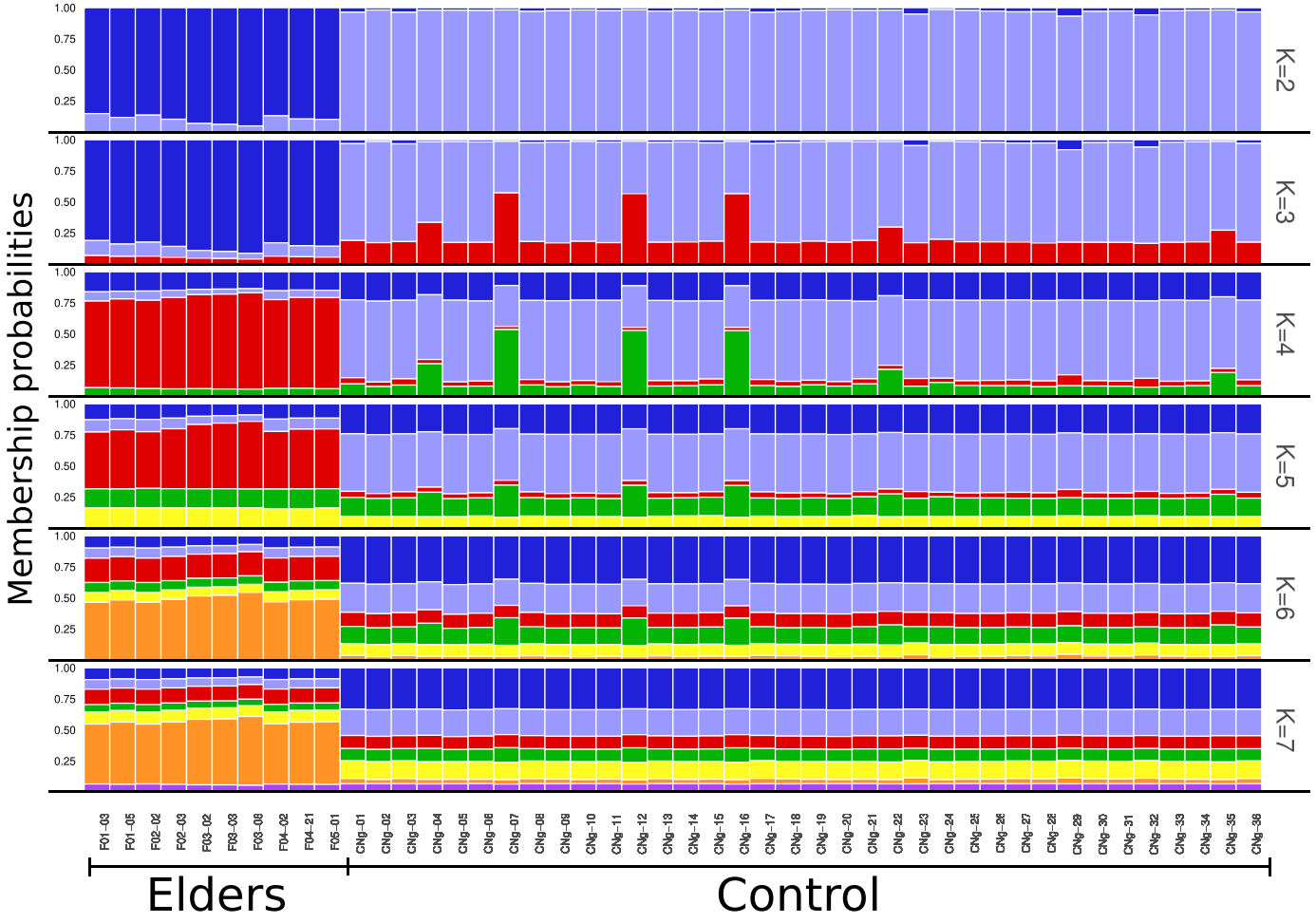

Fig. 3. Genetic cluster analysis between elders of each family and negative controls, considering the existence of 2 to 7 genetic groups (K). The bars above each individual indicate its membership probabilities on a scale of 0.0 to 1.0 considering each tested K. The number of colors presented in the chart is equivalent to the number of K.

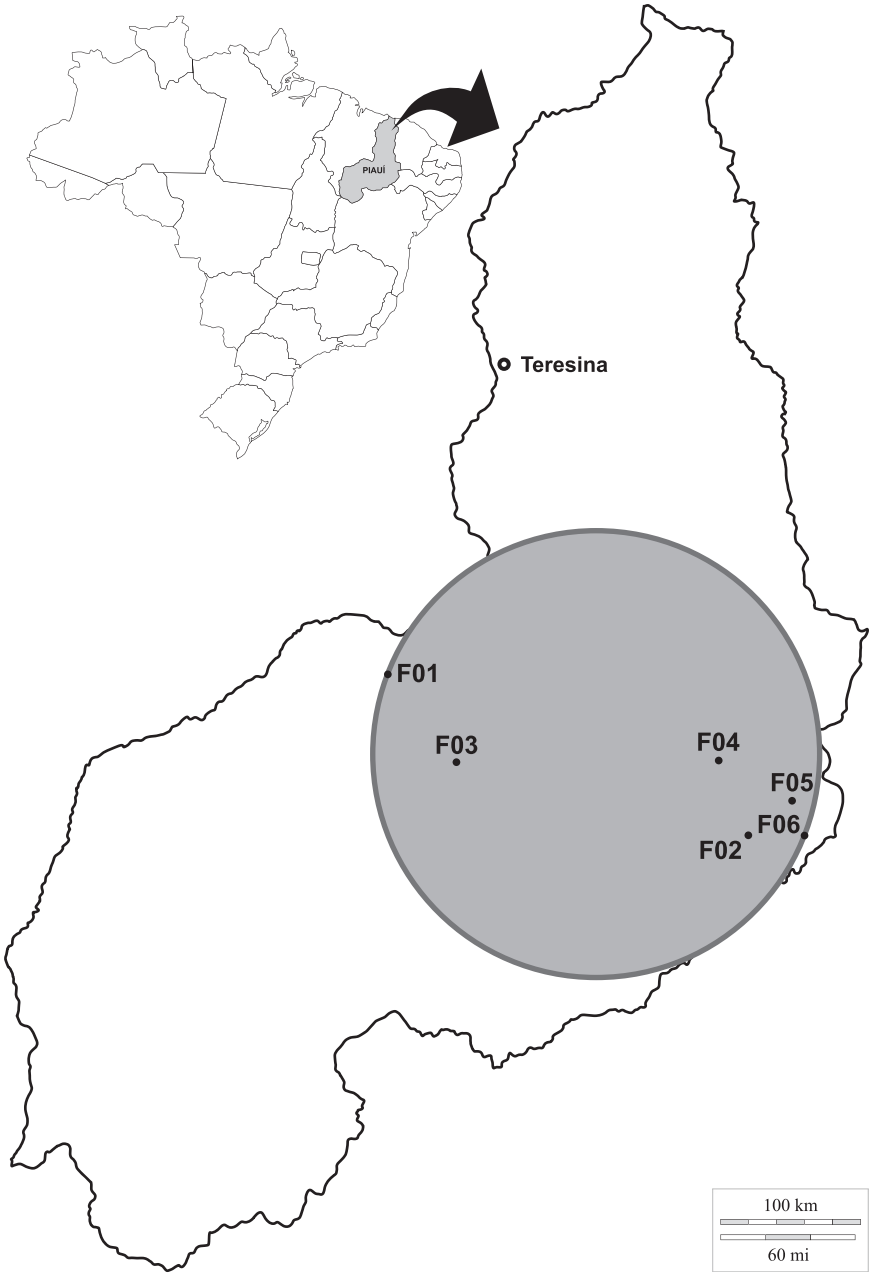

Fig. 4. Map of Brazil highlighting the state of Piauí with an approximation revealing the proximity of locations where each family resides. Geographical coordinates of cities: F01 (−7.087542083096411, −43.509134333430 56), F02 (−8.134889003994939, −41.14333225657599), F03 (−7.777304608346234, −43.137239317995984), F04 (−7.716868748179102, −41.34969456403206), F05 (−7.966926843069498, −40.872506199431974) and F06 (−8.141389765084043, −40.79823034193549).

increasing the rate of cases of autosomal recessive heteropathies that do not affect longevity and reproductive capacity [28]. In addition, it is well-known that the possible increase in autosomal recessive disorders in a region may be directly related to consanguineous unions, transmitting two copies of deleterious recessive alleles to the offspring [29,30].

The Brazilian Northeast is the region with the highest prevalence of consanguineous marriages in the country, mainly in the inland region, with an inbreeding coefficient 13 times higher in some northeastern populations than in the southern population [31–34]. Despite the knowledge about this inbreeding trend in the interior of the Northeast region and the existence of marriages between relatives in the examined family nuclei, these marriages did not contribute to increased consanguinity within the families (Table 1). Therefore, consanguineous marriages fail to justify the large number of patients with FLD identified in the backland of Piauí. The accuracy of inbreeding estimates (F) could be greater if biological samples from all individuals in the pedigree were available for DNA analysis. However, even in this case, no changes in the conclusion of the tests would not be expected. *I.e.*, the hypothesis of a high intrafamilial consanguinity would still be discarded due to the magnitude of the negative values found in F index. As an example, we can observe that the most negative values of the F coefficient were found in the families that presented the smallest sample size in the molecular analyses. In families with more samples analyzed, there is a tendency for the F-value to be closer to zero (constituting random marriages), but still negative. Also, we can conclude that the inbreeding analysis using molecular data corroborates with the pedigree, where most of the marriages were taken among non-relative people.

Based on the history of the disease and colonization of the region where the families reside, along with the gathered evidence, we attempted to determine any potential kin relationship. The kinship analysis of Queller and Goodnight (Fig. 2) revealed a high value for the variable r ($0.17 < r < 0.39$) in clusters of each family, since for first-degree relationships (full sibling) r-value is 0.5 and for half-siblings r-

value is 0.25. In contrast, when the negative control samples were grouped, the r-value approximated 0.0, representing a random mating population where the related individuals do not have a relationship. On pooling the patriarchs of each family, the r-value was 0.07, differing from the negative control and suggesting a certain degree of kinship between selected individuals [17].

This hypothesis was confirmed using Bayesian analysis, which indicated that elderly individuals were more genetically related than that expected by chance. The analysis tested these individuals with samples of individuals randomly selected from the local population (the control group). As the analysis did not consider any prior information regarding the individuals, the results undoubtedly demonstrated that elders shared a genetic history among them. We tested six scenarios where samples could be split into two to seven different genetic groups. Notably, the elders remained clustered within the same group and separated from the control group in all situations. These findings reinforce the hypothesis of a "founder effect" started in the past, bearing in mind that our goal was to confirm that these individuals share some level of coancestry rather than prove that they are close relatives.

5. Conclusions

Our data indicate that the evaluated families share a genetic link. Notably, all families carrying the identical mutation in the *LCAT* gene possibly share a common origin, suggesting a founder effect. Future studies need to employ molecular markers of the *LCAT* gene to explain the presence of the same mutation in families without evidence of consanguinity.

The discovery of six families with patients with FLD and several individuals carrying the mutation in the *LCAT* gene in the northeastern backlands, a region that lacks adequate health care for this extremely rare disease, provides a warning to health authorities. Monitoring at a genetics center should be made available, along with a multidisciplinary team, to minimize the disease impact and improve the quality of life for these individuals, with the possibility of diagnosing new cases in this region.

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Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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