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B-252 In Vitro Expression Analysis of Variants in the Upstream Region of Genes Related to Familial Hypercholesterolemia

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BACKGROUND: Familial hypercholesterolemia (FH) is commonly described as an autosomal dominant disorder caused mainly by variants in genes *LDLR*, *APOB*, and *PCSK9*, resulting in severe hypercholesterolemia. However, the substantial number of individuals who are clinically diagnosed but negative for known FH-causing variants indicates that relevant variants outside of frequently analyzed regions might exist. Considering the increasing number of promoter variants reported as likely to cause FH and the relevance of in vitro expression assays in the pathogenicity evaluation of these variants, this work aimed to examine the functionality of variants *LDLR* rs36218923-T and *APOB* rs934197-T, identified in a Brazilian FH cohort.

METHODS: Upstream fragments of *LDLR* (-861 bp to +85 bp) and *APOB* (-1460 bp to +127 bp) were PCR amplified from genomic DNA of two FH patients carrying *LDLR* rs36218923-T and *APOB* rs934197-T in heterozygosis and homozygosis, respectively. After linearization and PGK promoter removal of the pGL4.53 vector using Kpn I and Hind III restriction enzymes, fragments were cloned upstream of the Firefly Luciferase (luc2) coding region using the NEBuilder HiFi DNA Assembly Cloning Kit. Site-directed mutagenesis was also performed using partially overlapping primers with 3 ′-overhangs and Platinum SuperFi II DNA Polymerase to produce different constructs, including one containing *LDLR* rs879254375-G, which is a known FH-causing variant. All constructs were confirmed by Sanger sequencing. pGL4.53 constructs containing either wild type or variant alleles and pNL1.1.TK (control of transfection variation, expressing NanoLuc Luciferase) were co-transfected into HepG2 cells. Wild type constructs were considered positive controls, while promoterless pGL4.53 was considered negative control. Luciferase assays were carried out 48 h after transfection using the Nano-Glo Dual-Luciferase Reporter Assay System. After normalization of luminescence units of each assay (Firefly/Nanoluc), luciferase activity was determined as the mean of four transfections with the assay performed in triplicate.

RESULTS: The construct carrying *LDLR* rs36218923-T variant caused a significant mean reduction of luciferase activity (75,4% ± 2,9% SEM) over the promoterless construct relative to *LDLR* wild type construct. However, the construct harboring the *LDLR* rs879254375-G variant caused a more substantial reduction of luciferase activity relative to the wild type construct (91,2% ± 0,9% SEM). This reduction was consistent with shown by the previous study that reported *LDLR* rs879254375-G as a functional variant, which corroborates the methods adopted in this study. APOB rs934197-T was previously reported as functional based on a CAT assay using constructs of two tandemly arranged 30 bp fragments of *APOB* promoter, but in our study, the construct carrying the entire *APOB* promoter sequence and harboring rs934197-T variant did not cause a significant difference in mean luciferase activity compared to *APOB* wild type construct ($P = 0.564$). It is important to mention that our *APOB* upstream constructs had the additional variants rs617314-G, rs1560357-T, rs1625764-A, and rs1800481-G, which are considered benign because they occur at high frequency in the general population (0.82-1).

CONCLUSION: Our results suggest that *LDLR* rs36218923-T is likely to contribute to FH phenotype and emphasize the importance of performing in vitro expression assays in determining relevant upstream variants for FH molecular characterization.