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Proprotein convertase subtilisin/kexin type 9 genetic screening using the vervet (*Chlorocebus aethiops*) model

Thobile Ngqaneka[1,2] | Kenechukwu Obikeze[2] | Zandisiwe E. Magwebu[1] | Chesa G. Chauke[1]

[1]Primate Unit and Delft Animal Centre (PUDAC), South African Medical Research Council, Tygerberg, South Africa

[2]School of Pharmacy, University of the Western Cape, Bellville, South Africa

Correspondence
Thobile Ngqaneka, Primate Unit and Delft Animal Centre, South African Medical Research Council, P.O. Box 19070, Tygerberg, 7505, South Africa.
Email: thobile.ngqaneka@mrc.ac.za

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Abstract

Background: The proprotein convertase subtilisin/kexin type 9 (*PCSK9*) gene has come to prominence due to its reported function in the clearance of low-density lipoprotein cholesterol. The vervet monkey (*Chlorocebus aethiops*) was utilized to study the genetics of *PCSK9* gene.

Method: Sixteen vervet monkeys were selected to screen for possible *PCSK9* polymorphisms and to determine gene expression.

Results: Four *PCSK9* sequence variants (T112T, R148S, H177N and G635G) were identified and three of these variants (H177N, R148S, and G635G) were categorized as loss of function mutations. A decline in gene expression levels was also observed in animals harboring these three variants. Although the selected variants might have affected the level of gene expression in the selected animals, individual variation was also noticed in some of these individuals with the G635G variant.

Conclusion: Based on the findings obtained from this study, it is suggestive that the activity of *PCSK9* was hindered.

KEYWORDS
cardiovascular disease, low-density lipoprotein cholesterol, sequence variants, vervet monkey

1 | INTRODUCTION

Cardiovascular diseases (CVDs) remain one of the principal causes for mortality globally, and this includes several diseases such as ischemic heart diseases, venous thromboembolism, and stroke.[1] Central to the development of these CVD disorders is atherosclerosis, which occurs due to the accumulation of low-density lipoprotein cholesterol (LDL-C) in the inner layer of the arterial wall,[2] and the build-up of cholesterol in macrophages due to the insufficient elimination of LDLs, fats, and cholesterol by active high-density lipoprotein.[3,4]

The contribution of environmental factors (diet or smoking) to the development of atherosclerosis cannot be overplayed, however, genetic factors are more significant determinants of the risk of atherosclerotic CVD.[5] Additionally, the emergence of genome-wide association studies has revealed that genes play a substantial role in the development of CVDs.[6] The proprotein convertase subtilisin/kexin type 9 (*PCSK9*) gene has attracted interest due to its reported role in the clearance of LDLs.[7] This gene is expressed in several organs, particularly the liver, small intestines, and the kidney,[8] where it is suggested to post-transcriptionally regulate the number of cell surface LDL receptors (LDLR) thereby regulating the plasma levels of LDL-C.[9,10] The PCSK9 molecule is recognized as the main controller for LDLR activity and a viable drug target for the treatment of hypercholesterolemia.[8,11,12,13] A sequence variant (E670G) of *PCSK9* was reported to be a significant determining factor of plasma LDL-C levels and was linked to the development of severe coronary atherosclerosis[14] and large-vessel atherosclerosis stroke.[15]

Studies conducted after the discovery of PCSK9 further revealed the importance of sterol regulatory element-binding protein-2 (SREBP-2) in the regulation of cholesterol metabolism.[16-18] The SREBP-2 molecule is reportedly activated by low levels of intracellular cholesterol prompting the expression of LDLR[19,20] with the

increased levels of LDLR enhancing the clearance of LDL from circulation.[19,20] Concurrently, SREBP-2 can also encourage *PCSK9* expression, thereby promoting the breakdown of LDLR.[21,22] Therefore, this harmonized interaction of SREBP-2 induces the production of both LDLR and PCSK9 with the end goal of controlling LDL levels.[21,22]

Although there have been a larger number of studies investigating the genetics of PCSK9,[23-25] not many have studied the effects of its mutations on response to drug treatment. Therefore, the current study was aimed at acquiring *PCSK9* mutations that underscore heritable variations of lipoproteins, as opposed to replicating the exact genetic variants that are found in humans.[26] In this study, the vervet monkey model was utilized because of the similarities that appear to exist with humans in terms of the intravascular metabolism of cholesterol which are further enhanced by having an active cholesteryl ester transfer system.[27] Therefore, 16 vervet monkeys were utilized to investigate the possible presence of *PCSK9* polymorphisms and their impact on CVDs.

2 | MATERIALS AND METHODS

2.1 | Animal ethics and group selection

Ethics approval for the study was obtained from the South African Medical Research Council (SAMRC) Ethics Committee for Research on Animals (Ref: 11/18). Sixteen vervet monkeys used in the study were obtained from the Primate Unit and Delft Animal Centre (PUDAC) of the SAMRC. The monkeys were housed in single cages throughout the study with regular access to exercise cages and environmental enrichment. The closed indoor environment was maintained at a temperature of 24–26°C, 45% humidity, 15–20 air changes/h, and a photoperiod of 12 h as previously reported.[26] The monkeys were fed the same food content as previously reported.[28,29]

2.2 | Blood collection, DNA extraction, and quantification

The selected monkeys were sedated with Ketamine hydrochloride (10 mg/kg; Kyron Laboratories) anesthesia via intramuscular injection. Blood samples (2–4 ml) were obtained via femoral venipuncture into EDTA tubes. The DNA extraction procedure using the NucleoSpin® Blood kit (Macherey-Nagel) was followed as previously reported.[30] The DNA concentration and purity of the extracted samples were determined by measuring the absorbance at 260/280 and 260/230 nm in a Nanodrop 2000 spectrophotometer (Thermo Fisher).

2.3 | Bioinformatics and candidate gene selection

The *PCSK9* genome sequence was retrieved from the National Center for Biotechnology Information (NCBI), University of California Santa Cruz genome browser, and Ensembl databases, this gene was prioritized based on its reported functions in lipid metabolism. Primers were designed using the NCBI primer design tool (Table A1). The green monkey (*Chlorocebus sabaeus*) genome sequence from NCBI (accession number XM_007978607.1) was used as a reference for sequencing alignment since the captive-bred vervet (*Chlorocebus aethiops*) reference genome has not yet been annotated in the public domain. Sequencing results were analyzed using the following bioinformatics tools: Chromas lite software (https://technelysium.com.au/wp/chromas/), CLC DNA Workbench (CLC Bio), ClustaW2 (https://www.genome.jp/tools-bin/clustalw), and ExPASy translate tool (https://web.expasy.org/translate/). Mutation prediction tools such as Mutation-Taster® (http://www.mutationtaster.org/), SIFT (http://provean.jcvi.org/index.php), and PolyPhen 2 (http://genetics.bwh.harvard.edu/pph2/) were also used to determine the potential impact of each sequence variant.

2.4 | PCR amplification, purification, and sequencing analysis

The *PCSK9* gene was amplified by polymerase chain reaction (PCR) using GoTaq® green master mix (Promega). The PCR conditions adopted from[31] consisted of the following steps; 95°C for 5 min, followed by 30 cycles of 95°C for 30 s, 40–70°C for 30 s, and 72°C for 1 min. An extension period of 5 min at 72°C completed the procedure. The PCR products were subjected to gel electrophoresis (2%) to confirm the band size for each exon. The amplicons were further purified using Wizard® SV Gel and PCR clean-up system (Promega) and the purified PCR product was sent for bidirectional Sanger sequencing.

2.5 | Reverse transcription quantitative real-time PCR (RT-qPCR)

The PAXgene Blood RNA Kit (Qiagen) was used to isolate and purify intracellular RNA from whole blood collected in the PAXgene Blood RNA Tubes. The exact procedure previously reported for RNA extraction, purification, cDNA conversion[28,29] was employed. The RT^2 qPCR primer assay (Qiagen), designed for SYBR® Green-based qPCR detection (Applied Biosystems) was used for *PCSK9* (PPQ16344A). The qPCR standards were prepared using human liver total RNA (Takara) and samples were run in duplicates using Applied Biosystems universal cycling conditions; 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, and 60°C for 1 min. Since the SYBR green master mix was used, a melting curve was included in the run. The delta–delta ($2^{-\Delta\Delta Ct}$) method was used to calculate relative mRNA expression levels and further normalized using two housekeeping genes; *ACTB* (PPQ00182A) and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*: PPQ08645A).

2.6 | Statistical analysis

The gene expression analyses data generated in this study were presented as means ± SD using Delta Ct values for relative mRNA expression. Multiple comparison analysis was used to determine statistical significance between mutated and wild-type individuals using GraphPad Prism program. Statistical significance was calculated by using the Student's *t*-test, and a value of $p < .05$ was considered significant.

3 | RESULTS

Sanger sequencing analysis of the *PCSK9* gene indicated three sequence variants in exon 1, the first being a silent mutation (T112T) that occurred as the result of G336>A in one animal. The second was a homozygous missense variant (R148S, Table 1) that resulted from C442>A transversion in four animals and this variant was predicted to be a polymorphism by Mutation-Taster®. The third was identified as a missense variant (H177N) which affected eight individuals. In exon 10, a homozygous single nucleotide polymorphism (SNP)/silent sequence variant (G635G) was identified in 13 individuals. The impact of these sequence variants was correlated with relative gene expression. Although the expression findings were not significantly different ($p > .05$), the sequence variants identified in *PCSK9* gene (R148S, H177N, and G635G) further revealed that all affected animals had a decreased *PCSK9* expression when compared with the wild-types (Figures 1–3) However, within these variant carrying subjects, individual gene expression variability in correlation with LDL-C levels was observed.

4 | DISCUSSION

Sixteen vervet monkeys were screened for sequence variants that might predispose or shield them from developing CVDs. *PCSK9* gene was prioritized based on the knowledge that it plays a critical role in lipid metabolism and is known to regulate the levels of LDL-C. Sanger sequencing of the *PCSK9* gene in vervet monkeys revealed four sequence variants, with three being found in the first exon. One of these mutations is a missense variant (R148S) that resulted in a positively charged arginine being replaced by a polar serine. Mutation-Taster® predicted that this variant is a polymorphism, while other tools indicated a neutral or benign impact. The alignment of human *PCSK9* and the vervet monkey gene revealed that this variant (R148S) is found at codon 29 (R29S) in *Homo sapiens*. The first part of exon 1 of the human *PCSK9* gene is also referred to as the signal peptide, which covers the first 30 amino acids of the gene. The importance of this region allows the formation of three subsequent heterodimer proteins within three domains.[32] Miyake and colleagues identified a sequence variant in the same region as R29S, which was classified as being a minor or relatively common in individuals with low LDL-C levels. Concurrently in this study, the R148S (R29S: human) variant was identified in three individuals with relatively low LDL-C levels; and this suggested that the variant may fall in the category of loss of function (LOF) mutations.[33] According to the reference values of the SAMRC/PUDAC captive-bred vervet monkeys as well as those reported in literature, LDL-C levels above 2.75 mmol/L for both genders are considered high.[34,35] The individuals that harbored R148S variant had LDL-C values that were between 1.18 and 1.35 mmol/L, hence it is postulated that they may not be predisposed to CVD development. Another variant found in exon 1 of the vervet model was H177N (H58N in humans), which is located in the prodomain region (31–152 amino acids). This region is known to undergo self-induced cleavage while it remains bound to the rest of the molecule.[32] When the vervet sequence was aligned with human, the H177N/H58N variant, was predicted to be a polymorphism by Mutation-Taster® and five affected animals had relatively low LDL-C levels compared with the LDL-C reference (2.75 mmol/L). Additionally, there is a reported missense sequence variant (E57K), which is in close proximity to H58N identified in human subjects with low LDL-C which belongs to LOF category of mutations.[36] Although the vervet H177N variant and E57K are associated with low LDL-C levels, it would be premature to conclude they carry the same effect. The last novel variant to be identified in exon 10 of *PCSK9* is G635G (G516G: human). This homozygous variant is found in the region where the interaction with other cell surface proteins occurs.[12] It has been reported that the positively charged C-terminal domain of *PCSK9* gene tightly attaches itself to the negatively charged LDLR, resulting in the latter being locked in an open conformation.[37-39] This regular occurrence is enhanced by *PCSK9* gain of function mutations leading to increased LDL-C levels and predisposal to CVD development.[40] To circumvent the normal physiology of PCSK9, LOF mutations lead to more efficient LDL-C clearance due to decreased degradation of LDLR.[41] Furthermore, the monkeys with G635G had lower mRNA gene expression compared

TABLE 1 *PCSK9* identified sequence variants in the colony of captive-bred vervet monkeys

Genes	Exons	Nucleotide change	Amino acid change	Type of mutation	SIFT	PolyPhen 2	Mutation-taster®	Affected animals
PCSK9	1	G336A	T112T	Silent	ND	ND	ND	1
	1	C442A	R148S	Missense	Neutral	Benign	Polymorphism	4
	1	C529A	H177N	Missense	Neutral	Benign	Polymorphism	8
	10	T1905G	G635G	SNP	Neutral	Benign	DC	13

Abbreviations: DC, disease-causing; ND, not determined.

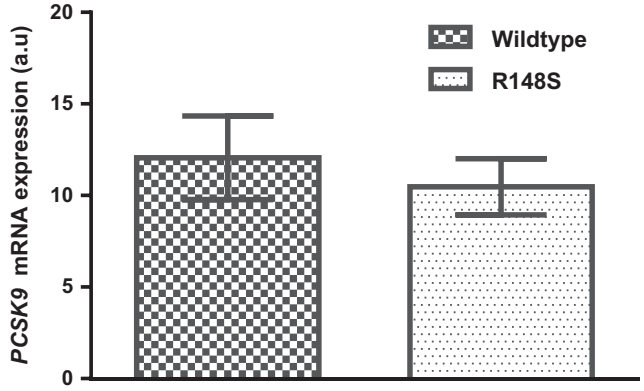

FIGURE 1 *PCSK9* mRNA gene expression between wild-type and animals with R148S variant. The data were expressed as mean ± SD using delta Ct values in arbitrary units (a.u.)

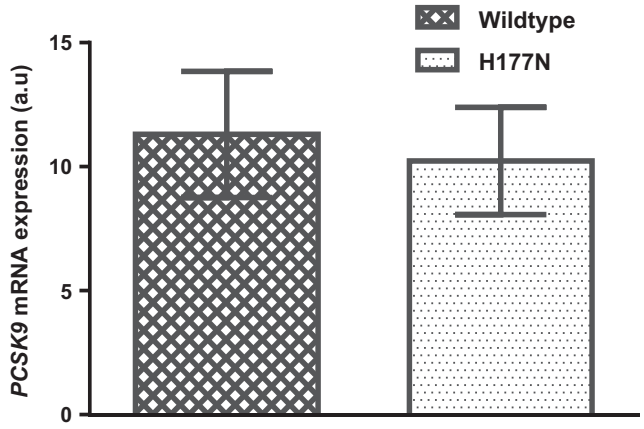

FIGURE 2 *PCSK9* mRNA gene expression between wild-type and animals with H177N variant. The data were expressed as mean ± SD using delta Ct values in arbitrary units (a.u.)

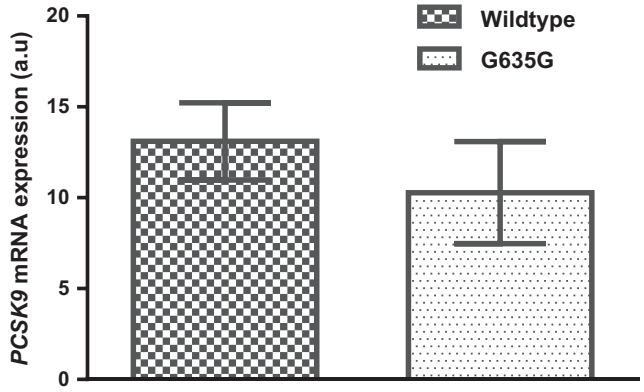

FIGURE 3 *PCSK9* mRNA gene expression between wild-type and animals with G635G variant. The data were expressed as mean ± SD using delta Ct values in arbitrary units (a.u.)

with the wild-type (p = .24, fold-change = 7.05; Figure 3), it is therefore postulated that this variant may play a role in offsetting the normal effect of PSCK9.

One of the reported reasons behind the inability of statins to effectively clear LDL-C in certain individuals is the ability to induce PCSK9 expression.[42] Therefore, this study also investigated the impact of identified sequence variants on the mRNA expression of *PCSK9*. Studies have also reported the importance of hepatocyte nuclear factor 1 α (HNF1 α), which is a key inducer of *PCSK9* gene expression.[43] This protein molecule is found 28 bp upstream from sterol regulator element (SRE-1), a critical sequence motif for PCSK9 transcription.[43] Therefore, it is hypothesized that the SRE-1/HNF1 α complex can be inactivated by sequence variants or by encouraging the depletion of HNF1 α leading to reduced expression.[42] Furthermore, the R29S variant mutation (vervets: R148S, see Table 1) is found just one amino acid away from the HNF1 α region, although this variant was predicted to be polymorphism there is a possibility that it played a role in attenuating gene expression. Consequently, the gene expression of *PCSK9* in all the animals with the variants was lower than the wild-type individuals for the three identified variants (R148S, H177N, and G635G; Figures 1–3). However, there were individual variations observed for G635G, which was correlated with LDL-C levels. For example, one individual had high LDL-C concentrations which resulted in high *PCSK9* gene expression when compared with the individual with low LDL-C.

Furthermore, the role played by the *LDLR* gene in the clearing of LDL-C is well documented, therefore it was of interest to determine its genetic variations in the captive-bred vervet monkey model. For comparison purposes, sequencing analysis of *LDLR* and *SREBP-2* (Table A2) was conducted concurrently with *PCSK9* due to the nature of their known interaction.[24,25] A sequence variant identified in *LDLR* (D274N; Table A2) was predicted to be deleterious by SIFT. This variant is found in the A7 molecule, which is critical for the proper functioning of LDLR as it is involved in the binding of LDL particles.[44] Furthermore, the mRNA expression of D274N carriers was also lower than the wild-types (Figure A1). According to the literature, the correlation between LDLR and PCSK9 is regarded as a counter-regulatory mechanism to maintain cholesterol homeostasis and constant LDL-C concentrations (Gu et al., 2015).[44] This means that individuals overexpressing PCSK9 are expected to have an increase in LDLR degradation, resulting in an increase of LDL-C plasma concentration. In this study, this phenomenon was observed between *PSCK9* (G635G) and *LDLR* (D274N) carrying individuals. The individual that had high *PCSK9* gene expression also showed decreased LDLR gene expression levels.

Should this be the case, the lipid pathway must devise a plan to counteract this drop in LDLR activity by synthesizing more LDLR molecules. That is where SREBP-2 comes in with its dual role in controlling the production of cholesterol while also upregulating PCSK9. An increase in cholesterol production leads to LDLR being also simulated to control the excess cholesterol. In this study, a disease-causing *SREBP-2* variant (P565P) was identified (Table A2). This variant was found to be located in the second transmembrane domain, a region reported to be within the essential functional domains covering exons 5–10 of *SREBP-2*.[45] Animals carrying the sequence variant had slightly elevated mRNA expression (p = .18,

fold-change = 0.67; Figure A2). Contrary, this P565P variant lowered the expression of *SREBP-2* in some of the affected individuals with high LDL-C concentrations. However, this individual variation was similar to the one observed for LDLR, which further confirms the role of SREBP-2 in regulating the expression of both PCSK9 and LDLR. Therefore, the predicted impact of the P565P variant supported the notion that silent mutations can also induce incorrect mRNA splicing leading to altered protein expression and enzymatic activity by affecting mRNA stability.[46,47]

This study was aimed at determining *PCSK9* sequence variants that play a critical role in the lipid pathways. The four identified *PCSK9* sequence variants consisted of two missense (R148S, H177N), one silent/synonymous (T112T) and one SNP (G635G). MutationTaster® predicted that G635G variant was disease-causing whereas the two *PCSK9* variants (H177N, R148S) were categorized as polymorphisms, and this was supported by a decline in gene expression in animals harboring these variants. However, individual variation was also observed for monkeys with G635G, which correlated with LDL-C levels. Furthermore, a deleterious mutation (D274N) identified in the *LDLR* gene was correlated with the inadequate LDL-C clearance that was observed in these vervet monkeys.

Based on the findings obtained from this study, it is suggestive that the activity of PCSK9 was hindered. This is supported by the identified variants which affected individuals with relatively low LDL-C levels, and further correlated with the uninterrupted LDLR function. However, it is possible that other underlying genetic mechanisms could have led to high LDL-C levels in certain individuals, and thus require further investigations to determine the exact impact of these variants in response to atherosclerosis drug therapy.

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CONFLICT OF INTEREST

The authors declare that there was no conflict of interest in the undertaking of this study.

DATA AVAILABILITY STATEMENT

The data that support the findings will be available in [repository name] at [DOI/URL] following an embargo from the date of publication to allow for commercialization of research findings.

ORCID

Thobile Ngqaneka https://orcid.org/0000-0003-4235-9970
Kenechukwu Obikeze https://orcid.org/0000-0002-3125-2257
Zandisiwe E. Magwebu https://orcid.org/0000-0003-4861-6018
Chesa G. Chauke https://orcid.org/0000-0003-3834-7467

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APPENDIX A

See Tables A1 and A2 and Figures A1 and A2.

TABLE A1 Designed primer pairs for *PCSK9*, *CETP*, *LDLR*, *APOB-100*, and *SREBP-2*

Gene	Exon	Forward sequence	Reverse sequence	Product size (bp)	Annealing temperature (°C)
PCSK9	1	CCACACCCTAGACGGTTTCC	AGATCGTGCCAAGCGAAGAG	659	60
	2	TGGGTCCGCATTTGGTAACT	GACATTGCGGACCCTTGAGT	749	61
	3	GGGACAAACCGAATTGCCAG	GGCAGAGCAAATGGATTCAGC	579	61
	4–5	CTAAAATGAGCCGACCAGCG	TCAAGTCTGCACTCCTTGGG	1192	66
	6	GCTGGGGTTGGCTAGTTTGA	GCTCAAGTGGTCAGTCATGC	1137	66
	7	ACCCGAGGGACATTCAGAGA	ACAGACCCTGACTGCTAAAGG	926	52
	8–9	CCTGGCCGATAAGACAGCAA	CCCATCCAGCACCCATTGAA	1105	66
	10	TGACGAGGGTGCTTGAGTTG	TTAGGCAAAGCACCTGCCAT	553	62
	11	CCCTGACCATCTGTTTGGCT	TGGATCTGAAGCAAGCGTGA	623	68
LDLR	1	ACTGGAGTGGGAATCAGAGC	CGTGCCATTACCCTACAAGTCT	581	64
	2	TCTTGGGTGCCTTCCTTGTG	TTTCCAGCCGCCATCATCAA	533	66
	4	ACTCAACCCAAAATAAGGACAGGA	TCGAAATCCACTTCAACACCT	779	60
	5	CACAGGGACCAACGAATGCT	TAACAGATGGGCCGCAAACA	217	58
	6	GGTCACGTCATGGTTGAGGA	ATATGCAGTCATGCCCCTGG	488	64
	9	CAGCAGGGCTATTTCCCAAGC	CAGTTTGGGCTTGTCCCAGAG	329	64
	10	TCAGGCTCACACATGGTTGG	CACGACCAGTTTTCCGCATT	388	60
SREBP-2	1	GGTTGTCGGGTGTCATGGG	TCCACTCCGCTGTTAAGGC	902	64
	2	TCCTGTCCTGTTGTCCTGGG	ACCTGCCATTCCTGAAGACGA	749	60
	3	CACTTGCCGTCCAGAAAGGA	CAACCAGTGCCTCCCTGAATC	570	–
	4	GTGTCTGGCTTTCCTGAGTCTT	AGACCCTCCCATACCTACTCAG	925	–
	5	AGGTCTGTGTTGAGGTTGCT	TTCACTTACCTTGAGCCTGGG	393	–
	6–7	GTGCTAGGGCTCTGCTCTTT	GTTTTTCTCCAGGCTAGGCCC	583	64
	8	TGGGGTGGGTGTAGACAATG	CCCTATCAGAAGCAAGCAGGT	968	64
	9	AGGCAAGCCCAAGTTCAGTC	TCAATGCTGTGAGTTGCCCC	1106	64
	10	AGGGATTCTTCCTGGGGGAT	TAGGGTGCAGAACTGGCAAAT	558	58
	11	TTGATCGTGCTGGAGAGAGC	GCTAGGTAAGTGCTCAGGGG	301	60
	12	CCGAGGGGAAAGTACAGCAG	TGGCTCCAAGGTATTCTCACC	581	–
	13	GTAGTTCTGGGCTTTGGTGGA	ACTTGGTCTAGGAGGAAAGGC	457	–
	14	CATGAGGTGGGCCTTGTGTA	ACTGATGTGATGAGGGGCTG	598	62
	15	GTGCTGGTCTATTCCCCTCC	GTTGTGCAGAGCAGGACACT	519	–
	16	CAGACGTGATGGTGAAGCCT	GGGACTCTGGGAGCCTAAGA	665	60
	18–19	TACGGGACAACAGAGGTTGC	TTTCTGCCCTGCCACCTATC	1189	–

TABLE A2 Identified sequence variants in the colony of captive-bred vervet monkeys

Genes	Exons	Nucleotide change	Amino acid change	Type of mutation	SIFT	PolyPhen 2	Mutation-taster®	Affected animals
LDLR	1	T56C	V19A	Missense	Neutral	Benign	Polymorphism	16
	6	G820A	D274N	Missense	Deleterious	Benign	DC	13
SREBP-2	9	C1695A	P565P	Silent	Neutral	ND	DC	5

Abbreviations: DC, disease-causing; PD, possibly damaging; ND, not determine; N/A, not applicable.

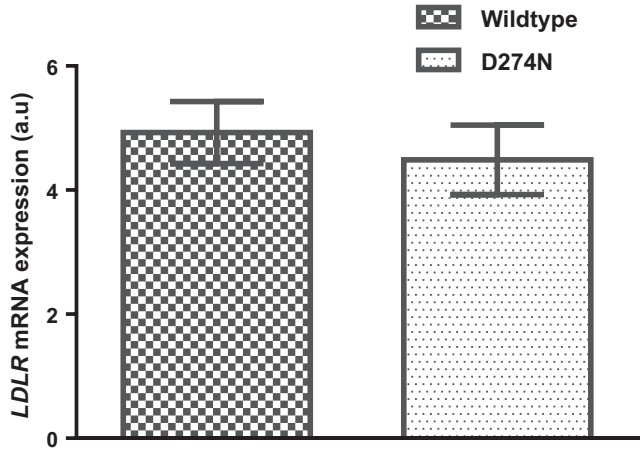

FIGURE A1 *LDLR* mRNA gene expression of between wild-type and animals with D274N variant. The data were expressed as mean ± SD using delta Ct values in arbitrary units (a.u.)

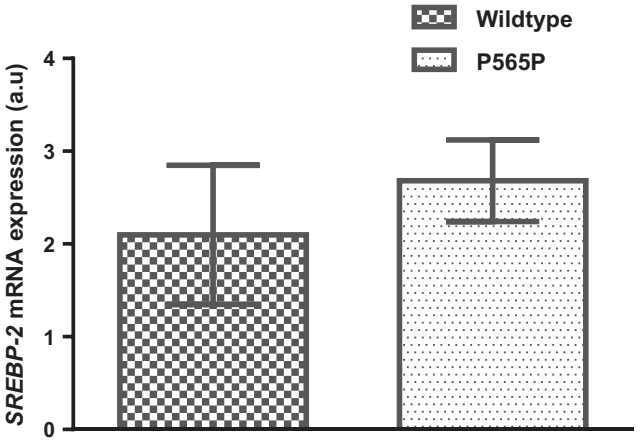

FIGURE A2 *SREBP-2* mRNA gene expression of between wild-type and animals with P565P variant. The data were expressed as mean ± SD using delta Ct values in arbitrary units (a.u.)