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# Aaptamine attenuates the action of statin on PCSK9 and LDLR expression as well as LDL-C uptake in human liver cells

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**ABSTRACT**

Atherosclerosis is characterized by the buildup of lipid deposits and plaque formation resulting from consistently elevated levels of LDL-cholesterol (LDL-C) in the blood. This study investigates the effects of aaptamine on the regulation of statin action concerning PCSK9 and LDL receptor (LDLR) expression, as well as LDL-C uptake in human liver cells. This research sought to assess the impact of aaptamine on the statin-induced elevation of PCSK9 expression, LDLR levels, and LDL-C uptake. The MTS assay was utilized to evaluate cytotoxicity. PCSK9 mRNA levels were quantified using real-time PCR, and protein expression was assessed through western blotting. Immunohistochemistry was utilized to evaluate LDLR levels and LDL-C uptake in hepatic cells. Results indicate that simvastatin elevated PCSK9 gene expression, achieving a maximum increase of fourfold. Co-incubation of aaptamine in simvastatin-treated cells significantly reduced PCSK9 gene expression. The co-treatment of cells with simvastatin and aaptamine resulted in a threefold increase in LDLR protein levels and LDL-C uptake rates. The results indicated that aaptamine reduced the effects of simvastatin on PCSK9 and LDLR expression, thereby enhancing LDL-C uptake by liver cells. This suggests that aaptamine may be a viable candidate for further development in the context of cardiovascular disease.



## 1. INTRODUCTION

Cardiovascular diseases encompass a range of disorders affecting the heart and blood vessels. Cardiovascular diseases remain the primary cause of global mortality, including conditions such as hypertension, coronary heart disease, stroke, heart failure, and peripheral vascular disease [1]. Deadly diseases are primarily attributed to a pathophysiological condition referred to as atherosclerosis [2]. The rising burden of cardiovascular diseases poses a significant challenge to achieving the United Nations Sustainable Development Goal (SDG) 3: Good Health and Well-being, particularly Target 3.4, which aims to reduce premature mortality from non-communicable diseases by one-third by 2030. Addressing CVD through prevention, early detection, and access to affordable treatment is essential to meeting this global health target. Furthermore, promoting heart-healthy environments and lifestyles aligns with other SDGs such as SDG 2 (Zero Hunger), SDG 11 (Sustainable Cities and Communities), and SDG 13 (Climate Action), recognizing the interconnection between health, nutrition, environment, and sustainable development.

Atherosclerosis is affected by several established risk factors, including elevated blood cholesterol and triglyceride levels [3], lipid metabolism disorders, smooth muscle cell migration and proliferation, oxidative stress [4], inflammation, and impaired endothelial function [5]. Atherosclerosis progresses in a defined manner at sites of blood vessel branching or curvature, leading to the formation of atherosclerotic lesions [6,7]. This process is triggered by the dysfunction of the endothelial lining of blood vessels and the

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alteration of increased levels of cholesterol, specifically low-density lipoprotein cholesterol (LDL-C) [8], resulting in the gradual accumulation of atherosclerotic plaque. The plaque manifests clinically at an advanced stage when the thickness of the overlying fibrous cap narrows the lumen of blood vessels, thereby restricting or potentially occluding blood flow to major organs, including the heart and brain [8], resulting in myocardial infarction and stroke [1].

Statins are effective agents for reducing lipid levels and are frequently utilized in the prevention of both primary and secondary cardiovascular disease [9]. Statins inhibit the enzyme 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase, responsible for converting HMG-CoA into mevalonic acid, a precursor for cholesterol synthesis in the liver [10,11]. These medications significantly decrease non-HDL-C levels, primarily by lowering LDL-C levels [12]. A substantial number of hypercholesterolaemic patients encounter challenges in tolerating elevated doses of statins [13].

Statins reduce cholesterol levels by inhibiting cholesterol synthesis and inducing the expression of the transcription factor SREBP2, which increases the transcription of Proprotein convertase subtilisin/kexin type 9 (PCSK9) and low-density lipoprotein receptor (LDLR) [14]. LDLR, located on the surface of hepatocytes, is essential for the absorption of circulating LDL-C. LDLR and LDL-C undergo internalisation through endocytosis, where LDL-C is degraded in lysosomes, and LDLR is subsequently recycled to the cell surface. Consequently, LDLR directly reduces the concentration of circulating cholesterols [15].

Proprotein convertase subtilisin/kexin type 9 is a serine protease synthesised in the liver [16]. PCSK9 acts as a natural inhibitor of LDLR through direct interaction with the LDLR-LDL-C complex, preventing its dissociation in endosomes, which results in the degradation of the complex in lysosomes. PCSK9's presence on the complex interferes with LDLR recycling [17]. An increase in PCSK9 diminishes the efficacy of statins in lowering LDL cholesterol levels [18].

The maritime environment, largely unexplored for medicinal applications, hosts a diverse array of organisms, including sponges and seaweeds, that coexist under challenging environmental conditions [19].

A prior investigation indicated that aaptamine, a compound derived from the marine sponge *Aaptos aaptos*, increased the transcriptional activity of the peroxisome proliferator response element (PPRE), which serves as a binding site for the transcription factor family of peroxisome proliferator-activated receptors (PPARs) located on the promoter of lipid-associated genes, including SR-B1 [20,21]. Additionally, the same compound was found to decrease the transcriptional activity of the PCSK9 promoter [22]. Interestingly, PPRE has also been demonstrated to be present on the PCSK9 promoter [23]. peroxisome proliferator-activated receptor α (PPARα) and PPARγ regulate the gene expression of PCSK9 by facilitating their interaction with specific PPREs [20,24]. Therefore, it is plausible to speculate that aaptamine may also reduce the transcriptional activity of PCSK9 via PPRE.

To date, no fundamental studies have explored the effects of aaptamine on statin-induced regulation of PCSK9, LDLR expression, and LDL-C uptake in liver cells. This study aimed to examine whether aaptamine can mitigate statin-induced PCSK9 overexpression of PCSK9. Specifically, it investigated the effects of co-treatment with aaptamine and statin on the expression levels of PCSK9 and LDLR genes, as well as the uptake of LDL-C by liver cells. Additionally, it sought to identify the transcription factors involved in mediating the action of aaptamine in modulating the effects of statin on PCSK9. Our recent research is referenced in Research Square: https://www.researchsquare.com/article/rs-5292389/v1.

## 2. MATERIALS AND METHODS

HepG2 cells were obtained from the American Type Culture Collection (ATCC, USA) and cultured in minimal essential medium (MEM) supplemented with 10% (v/v) foetal bovine serum (FBS), 1% (v/v) 1 mM sodium pyruvate, and 1% (v/v) penicillin (10,000 IU/ml)-streptomycin (10 mg/ml). Cells were cultured in a T25 flask and incubated at 37°C in a humidified incubator with 5% (v/v) $CO_2$.

### 2.1. Cytotoxicity screening using MTS cell proliferation assay

The CellTiter 96TM AQueous One Solution Cell Proliferation Assay was utilized as described in prior research [25]. Aaptamine, simvastatin, and their combined treatment were evaluated for *in vitro* cytotoxicity on HepG2 cells. A single treatment of aaptamine was prepared at serial dilution concentrations ranging from 250 to 2,000 µM in a 20% (v/v) DMSO solution. Subsequently, 5 µl of aaptamine was loaded into the wells in triplicates, achieving final concentrations of aaptamine at 12.5 to 100 µM with 1% DMSO. Aaptamine was prepared for co-treatment at serial dilutions of 250–2,000 µM, alongside 200 µM simvastatin in a 20% (v/v) DMSO solution. Subsequently, 5 µl of each diluted solution was loaded into the wells in triplicates, resulting in a final co-treatment concentration of simvastatin at 10 µM and aaptamine at 12.5–100 µM. Cells treated with 1% (v/v) DMSO functioned as the negative control, whereas wells containing only complete medium without cells acted as the background. Following the treatment, the microplate underwent incubation for 24 hours at 37°C, with 5% (v/v) $CO_2$ in 95% humidity. Following a 24-hour incubation period, 20 µl of MTS solution was introduced into each well, with an additional incubation of 4 hours at 37°C in a 5% (v/v) $CO_2$ incubator. Cell viability was assessed at 490 nm using a Spark multifunctional microplate reader.

### 2.2. Quantitative real-time PCR

HepG2 cells were utilized to extract total cellular RNA, subsequently lysed with TRIzol reagent from ThermoFisher. The real-time polymerase chain reaction (qPCR) was performed utilizing the iTaqTM Universal SYBRTM Green One-Step Kit (Bio-Rad) master mix, adhering to the manufacturer's guidelines. The gene primers used in this study are presented in Table 1. Each reaction comprised 150 nanograms of RNA subjected to DNase treatment, 10 microliters of a 2x SYBRTM Green qPCR reaction mix from BIORAD, 0.6 microliters of 10 millimolar concentrations of both forward and reverse primers (as detailed in Table 1), 0.25 ml of iScript reverse transcriptase for one-step qPCR, and nuclease-free water to achieve a total reaction volume of 20 ml. Each sample was analyzed three

**Table 1.** The nucleotide sequences of forward and reverse primers used in RT-PCR [24].

| Primer | Oligos | Sequences (5'-3') |
|---|---|---|
| PCSK9 | Forward | GGCAGGTTGGCAGCTGTTT |
| | Reverse | CGTGTAGGCCCCGAGTGT |
| β-actin | Forward | TCACCCTGAAGTACCCCATC |
| | Reverse | CCATCTCTTGCTCGAAGTCC |

times. The one-step real-time PCR was conducted utilizing the CFX6 Real-Time PCR Detection System from Bio-Rad. The mRNA copy numbers of the target were standardized using the housekeeping gene β-actin [24].

#### *2.2.1. PCR efficiency*

A series of 5-fold dilutions of the positive control sample was prepared and subjected to RT-PCR with optimized annealing temperature. The Cq values were plotted against the log of starting concentration, and a best-fit line was generated. PCR efficiency (E) can be calculated as E=10 (-1/slope). The percentage of efficiency = (E-1) × 100%. Therefore, assay efficiency should range from 90% to110%. Coefficient of determination (R2) for standard curve should also be R2 > 0.98.

In order to determine the relative quantification of gene expression, the ratio between the reference gene (RG) and gene of interest (GOI) can be calculated. The method used to express the foldchange of the RG and GOI is called the delta delta Cq method ($2^{-\Delta\Delta Cq}$), where 2 is derived from 1+efficiency (efficiency assumed as 1). The difference between Cq values of GOI and RG is calculated, so as to remove any errors or sample variation in multiple steps. This generates a ΔCq value for all samples, which is then compared back to a control sample to generate the ΔΔCq [26].

### 2.3. Protein extraction and western blot analysis

The study involved the extraction of cytoplasmic proteins from HepG2 cells using the NE-PER Nuclear and Cytoplasmic Extraction Kit, following the manufacturer's guidelines. The quantification of proteins was performed using the Bradford Assay, which evaluated the concentration of cytoplasmic proteins with a Quick Start™ Bradford 1x dye reagent and a bovine serum albumin protein standard set. The SPARK multifunctional microplate reader measured absorbance at 590 nm. A 10% (v/v) resolving gel and a 5% (v/v) stacking gel were utilised for SDS-polyacrylamide gel electrophoresis. Proteins were transferred from gels to membranes using Bio-Rad Trans-BlotTM Turbo™ Transfer System RTA Transfer Kits. Protein bands were identified utilising the PierceTM DAB (3,3'-diaminobenzidine tetrahydrochloride) Substrate Kit. The density values of the target protein bands were analysed utilising ImageJ software.

### 2.4. LDL-C uptake and immunofluorescence staining of LDLR and PCSK9

The uptake of LDL-C by LDLR was assessed utilizing an LDL uptake assay kit (Abcam™). The kit employed a fluorescent probe to quantify LDL uptake in cultured cells, utilizing human LDL conjugated with DyLight™ 550. A secondary antibody conjugated to DyLight™ 488 and a polyclonal antibody specific to LDL receptors were utilized as outlined in the previously described method [24]. Upon completion of the 24-hour treatment with aaptamine and simvastatin, the culture media in the 96-well plate was removed and replaced with 100 µl/well of LDL-Dylight™ 550 working solution. The LDL-DyLight™ 550 working solution was prepared by diluting the substance 1:100 with MEM culture media. In a similar manner, the rabbit anti-LDLR and anti-PCSK9 primary antibodies, along with the goat anti-rabbit IgG secondary antibody conjugated to DyLight™ 488, were individually diluted with TBST at a ratio of 1:100 to prepare the antibody solutions. FITC-labelled LDLR and PCSK9 were observed and documented using a Nikon Eclipse Ti2-E fluorescence microscope at excitation and emission wavelengths of 485/535 nm.

### 2.5. PCSK9 promoter-reporter plasmid

PCSK9 promoter, measuring 1.8 kb and located from –1,711 to –94 was used in this study. It encompasses many CAEs essential for the control of the PCSK9 gene transcription [27]. To delineate the regulatory regions of the PCSK9 promoter that mediated the action of aaptamine on statins in regulating PCSK9 transcription, seven 5' end deletion constructs of the PCSK9 promoter, designated as D1 (–1711/–94), D2 (–1214/–94), D3 (–709/–94), D4 (–440/–94), D5 (–392/–94), D6 (–351/–94), and D7 (–335/–94) [27], were transiently transfected into HepG2 cells. MatInspector software [28] was used to determine transcription factor binding sites on the PCSK9 promoter fragments.

### 2.6. Transient transfection and luciferase assay

Cells were seeded onto a 96-well transparent bottom plate at a density of $4.0 \times 10^5$ cells per well and left to incubate overnight. Transient transfection assay was carried out using Lipofectamine™ Plus Reagent (Invitrogen) according to the manufacturer's instruction. Solution A comprised 1.5 µl of Lipofectamine™ LTX reagent diluted in 25 µl of Opti-MEM™ serum-free medium. To prepare Solution B, 0.5 µl of PLUS™ reagent, 2 µg of PCSK9 promoter-luciferase plasmid, and 0.5 µg of pRL-TK plasmid were diluted in 25 µl of Opti-MEM™. Both solutions were combined and incubated for 30 minutes at room temperature to produce Solution C. After discarding the medium in the 96-well plate, cells were rinsed twice with PBS before adding 90 µl of Opti-MEM™ to each well. Subsequently, 10 µl of solution C was introduced into each well and incubated for 5 hours for transfection to take place. The old medium was then replaced with Opti-MEM™ for a single treatment containing aaptamine (100 µM), simvastatin (10 µM), and for the co-treatment containing simvastatin (10 µM) with aaptamine (100 µM). The untreated cells were incubated with 1% (v/v) DMSO, which served as the negative control. Cells were then placed in a 5% CO2 incubator at 37°C for 24 hours.

After treatment, the activity of the PCSK9 promoter was measured using the DualGlo® Luciferase Assay System following the directions provided by the manufacturer. First, 90 µl of Dual-Glo® Luciferase Reagent was added to each well and allowed to incubate for 10 minutes at room temperature under

low light conditions. The firefly luminosity was quantified using the GloMax® Multi Detection System equipped with an EX450 optic filter. Subsequently, 90 µl of Dual-Glo® Stop & Glo® reagent was introduced into each well and allowed to incubate at ambient temperature for an additional 10 minutes. *Renilla* luminescence was then measured using the same method as described before. To determine the relative fold change of PCSK9 promoter activity, the firefly luciferase activity of each transfection was normalised against the *Renilla* luciferase activity.

### 2.7. Statistical analysis

Data were presented as mean ± standard deviation (SD). Multiple groups were analysed with one-way analysis of variance using SPSS version 25 where means were separated with Duncan's Multiple Range Test at $p$-value < 0.05. Student's $t$-test was carried out to compare the statistical significance of means between a test sample and control group with Microsoft Excel. A nominal $p$-value of less than 0.05 indicates statistical significance.

## 3. RESULTS

### 3.1. MTS assay

The HepG2 cell line, originating from human hepatocellular carcinoma, served as the model system for this study. It was essential to establish that aaptamine demonstrated no cytotoxic activity against the cells. The MTS assay was conducted to evaluate the cytotoxic effects of aaptamine, statin and their combination on the HepG2 cell line.

As shown in Figure 1, lovastatin reduced cell viability by 2% compared to the control at 0.25 µM. However, at higher concentrations, cell viability exceeded that of the control. The highest cell viability (128%) was observed at 6 µM lovastatin, before decreasing to 112% at the highest concentration used (10 µM). For mevastatin, cell viability remained above 89% at all tested concentrations. The lowest viability was observed at 6 µM, while the highest (102%) occurred at 0.5 µM. At the highest tested concentration (10 µM), mevastatin reduced cell viability to 93% compared to the control.

Interestingly, simvastatin exhibited its lowest cell viability (97%) at 6 µM. At 10 µM, cell viability increased to approximately 103%, while the highest viability (112%) was observed at 1 µM. These results indicate that none of the three compounds reduced cell viability below 89%, suggesting a lack of significant cytotoxicity. Since simvastatin showed the least cytotoxicity and growth effect on HepG2 cells at 10 µM, it was selected for further experiments.

In order to determine the cytotoxicity effect of the combination of aaptamine and simvastatin, HepG2 cells were treated with various concentrations of aaptamine (12.5–100 µM) and fixed concentration of simvastatin at 10 µM.

The single treatment of aaptamine (12.5–100 µM) did not produce any significant change in cell viability on HepG2 cells when treated for 24 hours as compared to untreated control which indicates that aaptamine did not show any cytotoxicity effect on the cells, which in agreement to a previous study [21,29]. Co-treatment of 10 µM simvastatin with various concentrations of aaptamine (12.5–100 µM) also did not exhibit any significant change, with the lowest percentage of cell viability was observed at 12.5 mM with 95% (Fig. 2).

### 3.2.. Aaptamine suppresses the expression of the PCSK9 gene in HepG2 cells

HepG2 cells were seeded into 25 $cm^2$ cell culture flasks and allowed to grow until 70%–80% confluency. Prior



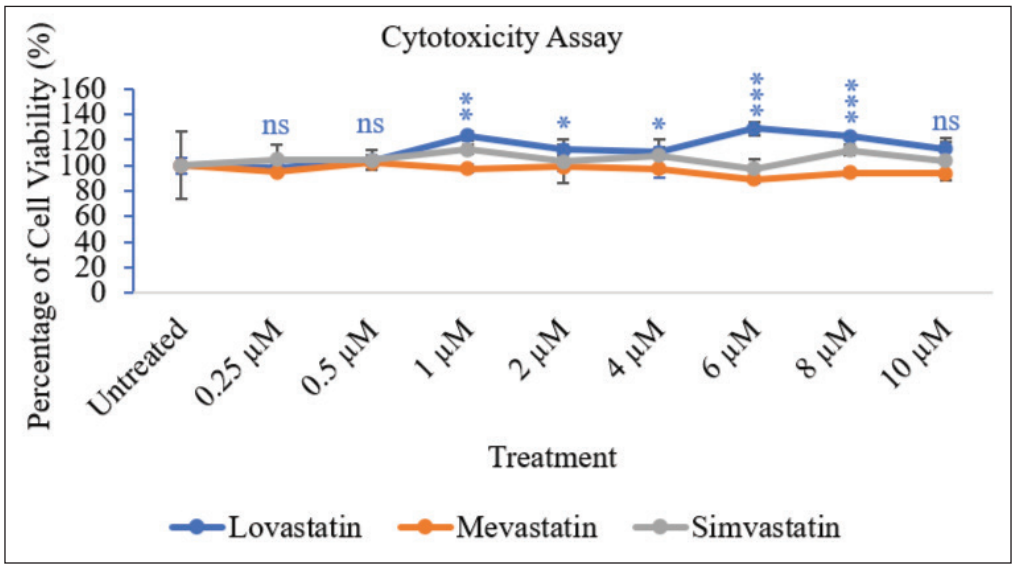


**Figure 1.** Percentage of cell growth after being treated with lovastatin, mevastatin, and simvastatin. HepG2 cells were treated with statins at concentrations ranging from 0.25 µM to 10 µM for 24 hours. The value in the untreated control was assigned as 100%, and the value in the treated samples was relative to the value of the untreated control. Data presented as mean ± SD with $n = 3$. * Indicates a significant difference between untreated control and treatment among the lovastatin samples (Student's $t$-test, $p < 0.05$). However, mevastatin and simvastatin did not show any significant difference between untreated control and treatment at any concentration. ns = not significant.

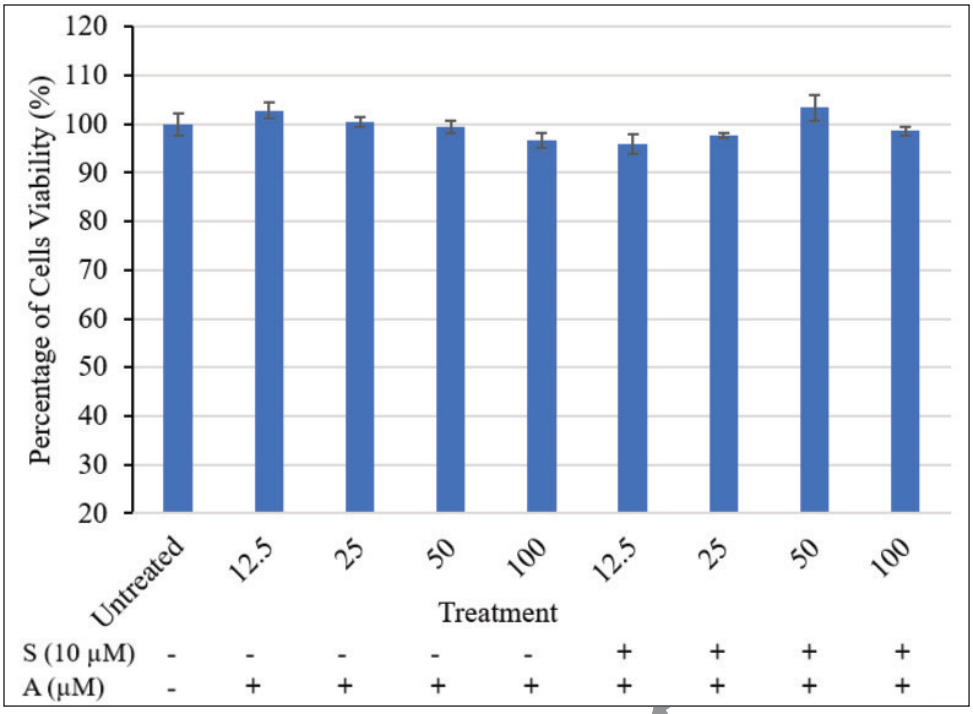


**Figure 2.** Percentage of cell growth after being treated with single treatment of aaptamine (12.5–100 µM) and the co-treatment of simvastatin (S) and aaptamine (A). HepG2 cells were co-treated with 10 µM simvastatin and various concentrations of aaptamine ranging from 12.5 µM to100 µM for 24 hours. The value in the untreated control was assigned as 100%, and the value in the treated samples was relative to the value of the untreated control. Data presented as mean SD with $n = 3$. Single treatment of aaptamine and co-treatment of simvastatin with aaptamine did not show any significant difference compared to untreated control (Student's $t$-test, $p < 0.05$).



to treatment with aaptamine and/or statin, the spent medium was removed, and the cells were washed twice with PBS. The medium was then replaced with fresh MEM containing 0.5% (v/v) FBS supplemented with either aaptamine, simvastatin or a combination of both compounds. To evaluate the effect of aaptamine on the action of statin in regulating PCSK9 mRNA expression, HepG2 cells were treated for 24 hours with either aaptamine at concentrations ranging from 12.5 to 100 µM or simvastatin at a fixed concentration of 10 µM, or a combination of both. In addition, cells were treated with 20 µM of berberine sulfate as a positive control and 1% (v/v) DMSO as a negative control.

Figure 3 demonstrates that aaptamine markedly decreased PCSK9 mRNA expression to below 7% across all concentrations when compared to the untreated control (1% (v/v) DMSO). Aaptamine at 12.5 µM resulted in a maximum reduction level of 99% compared to the control. In contrast, treatment with 10 µM simvastatin resulted in a significant 2.9-fold increase in the mRNA expression of PCSK9 compared to the untreated control. When cells were cotreated with simvastatin and aaptamine (at concentrations of 12.5–100 µM), the mRNA expression of PCSK9 was significantly reduced compared to the untreated control, with 50 µM aaptamine resulting in the lowest levels of PCSK9 mRNA at 6% of the control. The findings demonstrate that aaptamine reduced the inducible impact of statin on PCSK9 gene expression.

### 3.3. Aaptamine attenuates the effect of statin on the protein expression of the PCSK9 in HepG2 cells

As shown in Figures 4 and 5, two protein bands were present when the membrane was probed with antibody against PCSK9 which represent mature and premature PCSK9 protein with the size of 72 kDa and 63 kDa, respectively, which correspond to the previous work [30,31]. Corresponding to level of mRNA expression, aaptamine significantly downregulated the protein expression of both premature and mature PCSK9 (Fig. 4) as compared to the untreated control (1% (v/v) DMSO). The mature protein expression of PCSK9 was significantly downregulated between 45%–60% when treated with aaptamine with the lowest level of expression when 50 µM aaptamine was used to treat the cells. The premature PCSK9 protein expression was drastically decreased to between 6% and 18% of control, with aaptamine at 25 µM and 50 µM producing the lowest level at 6% whereas aaptamine at 12.5 µM and 100 µM reduced premature PCSK9 protein level to 18% and 13%, respectively.

As shown in Figure 5, there was a 4-fold (413.488 ± 0.0196) increase in mature PCSK9 protein expression as compared to the untreated control (100 ± 0.0263) when the cells were treated with simvastatin 10 µM. However, interestingly When HepG2 cells were treated with a combination of simvastatin and aaptamine, the protein expression of both premature and mature PCSK9 was significantly downregulated as compared to simvastatin alone. The level of PCSK9 was even lower than to that of untreated controls (Fig. 5). Specifically,

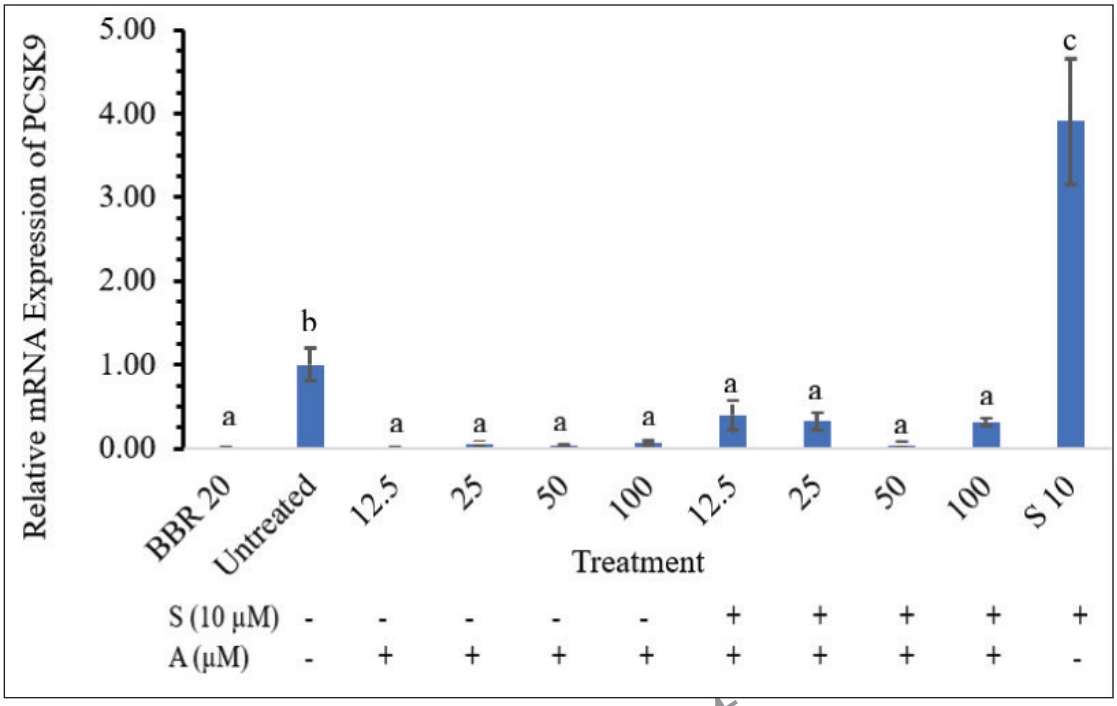


**Figure 3.** Relative mRNA expression of PCSK9 after being treated with a serial dilution of aaptamine (concentrations of 12.5–100µM) and Simvastatin 10 µM for 24 hours. The mRNA expression on the co-treatment of simvastatin (10 µM) with aaptamine (12.5–100µM) was also carried out. HepG2 cells were also treated with 20 µM of berberine sulphate as positive control and 1% DMSO as Untreated control for 24 hours. The value in the untreated control was assigned as 1.00, and the value in treated samples was relative to the value of the untreated control. Data presented as mean SD with $n = 3$. The different superscripts in the various treatments differ significantly ($p < 0.05$).



co-treatment with simvastatin and aaptamine significantly decreased to the PCSK9 mature protein expression between 16% and 49% than that of untreated control with co-treatment with 10 mM simvastatin and 25 µM aaptamine produced the lowest levels of mature PCSK9. Aaptamine at 50 µM and 100 µM exhibited a reduction of PCSK9 protein level by 81% and 80% of control, respectively. Premature PCSK9 protein expression was also downregulated dose-dependently during the co-treatment, however lower than to that of mature PCSK9 protein, ranging from 47% to 72% of control.

### 3.4. Aaptamine attenuates the action of statin in inducing the uptake of LDL-C

To assess the impact of co-treatment with aaptamine and statin on LDLR protein levels, HepG2 cells received either a single treatment of simvastatin (10 µM), aaptamine (12.5–100 µM), or a co-treatment of simvastatin with varying concentrations of aaptamine.

Figure 6 illustrates that immunohistochemistry staining using an antibody against LDLR revealed a higher intensity of FITC-stained HepG2 cells in those treated with aaptamine compared to the untreated control. This indicates an increased presence of LDLR on the cell surface in aaptamine-treated cells. Aaptamine elevated LDLR protein levels by 1.84-fold (184.4 ± 24.48) at a concentration of 12.5 µM, with a gradual decline observed; nonetheless, at the highest concentration of 100 µM, levels remained 1.25-fold (125.3 ± 4.938) higher than the control (100 ± 14.88). Statins, which inhibit 3-HMG-CoA reductase, reduce cholesterol levels by blocking cholesterol synthesis. Statins have been shown to increase LDLR mRNA levels and enhance the clearance of plasma cholesterol. Statin therapy has notable drawbacks, including medication intolerance and increased plasma levels of PCSK9. Statins increase the expression of both PCSK9 and LDLR, which diminishes the effectiveness of elevated LDLR levels in lowering plasma LDL-cholesterol levels [32].

The presence of LDLR protein on the cell surface of HepG2 cells increased by 1.16-fold (116.5 ± 9.278) compared to the control when treated with simvastatin, although this level remained lower than that observed in aaptamine-treated cells (Fig. 6). Co-treatment of cells with statin and aaptamine resulted in a significant increase in LDLR protein levels on the cell surface across all concentrations (Fig. 6). The maximum expression of LDLR protein occurred at 12.5 µM of aaptamine, exhibiting a 2.36-fold (236.8 ± 21.17) increase compared to the control. This was followed by 25 µM, which showed a 2.28-fold (228.9 ± 24.70) increase, 50 µM with a 2.17-fold (217.0 ± 24.54) increase, and 100 µM demonstrating a 1.52-fold (152.4 ± 12.07) increase relative to the untreated control.

The intensity of FITC-stained HepG2 cells indicates a significant dose-dependent reduction in the surface levels of PCSK9 protein following treatment with aaptamine, as shown in Figure 7. The lowest observed level was 39% compared to the untreated control at a concentration of 50 µM. Simvastatin

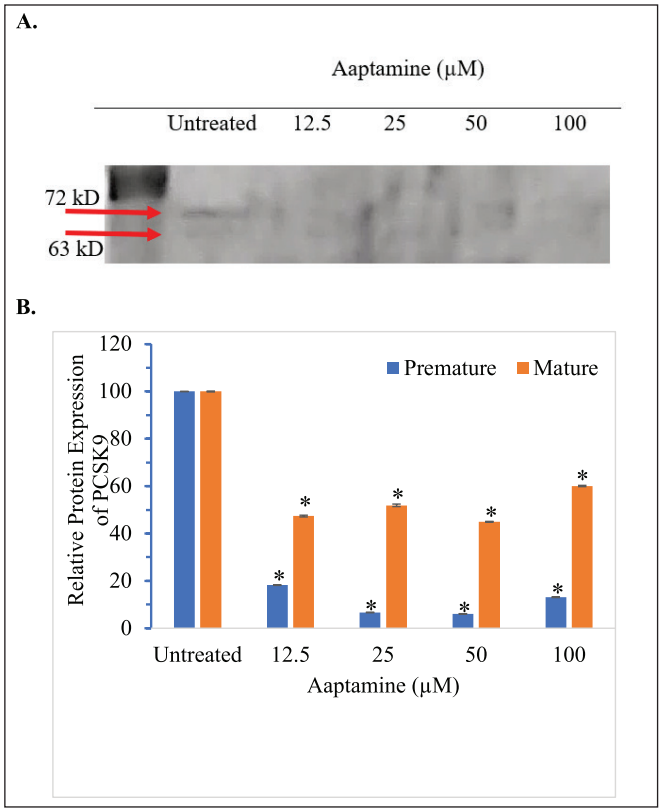


**Figure 4.** The level of PCSK9 protein expression. HepG2 cells were treated with single treatment of aaptamine (12.5–100 μM) for 24 hours. After protein extraction, a western blot was then carried out (A). The values in the untreated control were assigned as 100%, and the values in the treated samples were relative to the values of the untreated control. * Indicates a significant difference between control and treatment (Student's *t*-test, $p < 0.05$) (B).

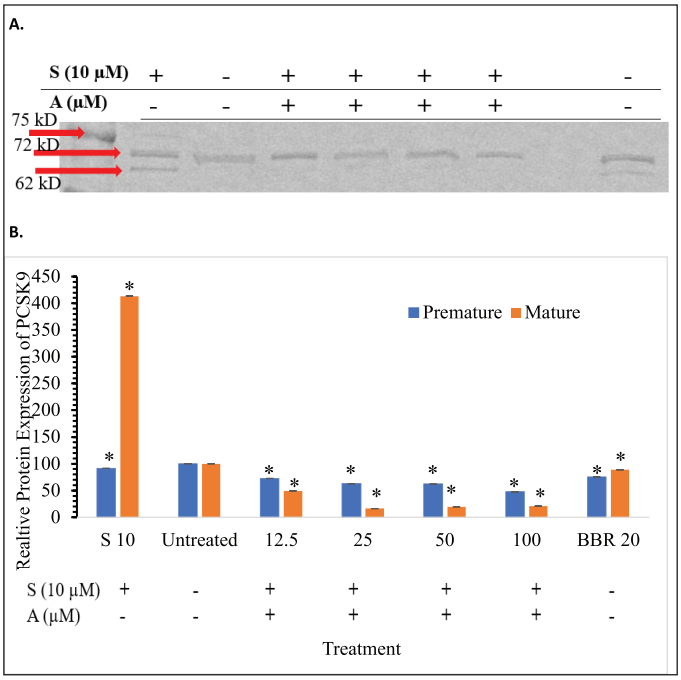


**Figure 5.** PCSK9 protein expression when HepG2 cells were cotreated with simvastatin (S) (10 μM) and aaptamine (A) (12.5–100 μM). HepG2 cells were also treated with 20 μM of berberine sulphate as positive control and 1% (v/v) DMSO as untreated control for 24 hours (A). The protein expression of both premature and mature PCSK9 in the untreated control group is assigned as 100%, and the values in the treated samples were relative to the value of the untreated controls. * Indicates a significant difference between control and treatment (Student's *t*-test, $p < 0.05$) (B).



resulted in a 1.49-fold (149.0 ± 9.752) increase in PCSK9 protein levels, correlating with mRNA and total protein content levels. In co-treated cells, aaptamine reduced the statin-induced increase in cell surface PCSK9 levels (Fig. 7). The co-treatment of cells with statin and 100 μM aaptamine resulted in the lowest level of PCSK9 protein, demonstrating a 43% reduction compared to the untreated control. Nonetheless, this reduction remained comparatively greater than that of aaptamine alone.

Figures 6 and 8 illustrate a significant correlation between LDLR expression and LDL-C uptake. The uptake of LDL-C significantly increased when cells were treated individually with either aaptamine or statin, although the inducible effect of statin remained lower than that of aaptamine. The co-administration of aaptamine and statin led to an enhanced uptake of LDL-C, surpassing the effects observed with either agent alone. The maximum increase was recorded at 3.22-fold (321.9 ± 29.59) when cells were treated with statin and 25 μM aaptamine. The highest rates of LDL-C uptake were observed at 1.74-fold (173.8 ± 44.54) and 1.19-fold (119.8 ± 36.42) increases compared to the untreated control when cells were treated with 100 μM aaptamine and 10 μM statin, respectively.

### 3.5. Identification of the regions of PCSK9 promoter that mediate the inhibitory action of aaptamine on statin in regulating PCSK9 transcriptional activity

PCSK9 promoter, measuring 1.8 kb and located from –1,711 to –94 was used in this study. It encompasses many CAEs essential for the control of the PCSK9 gene transcription [27]. To delineate the regulatory regions of the PCSK9 promoter that mediated the action of aaptamine on statins in regulating PCSK9 transcription, seven 5' end deletion constructs of the PCSK9 promoter, designated as D1 (−1711/−94), D2 (−1214/−94), D3 (−709/−94), D4 (−440/−94), D5 (−392/−94), D6 (−351/−94), and D7 (−335/−94) [27], were transiently transfected into HepG2 cells. Subsequently, the cells were treated with either aaptamine, simvastatin or their combination for 24 hours. Based on our previous results, aaptamine at the concentration of 100 μM, simvastatin at 10 μM, and the co-treatment of simvastatin at 10 μM and aaptamine at 100 μM were used to treat cells for 24 hours. The promoter activity was subsequently assessed using a dual-glo luciferase assay.

Figure 9 illustrates the differential effects of aaptamine on the promoter activity of seven promoter constructs, attributable to the presence of various binding sites for distinct transcription factors. Aaptamine significantly reduced the transcriptional activity of D1, D2, D3, D4, D5, and D6 promoter fragments by 46.7%, 63.7%, 23.2%, 70.3%, 43.0%, and 7.3% of control levels, respectively. However, aaptamine

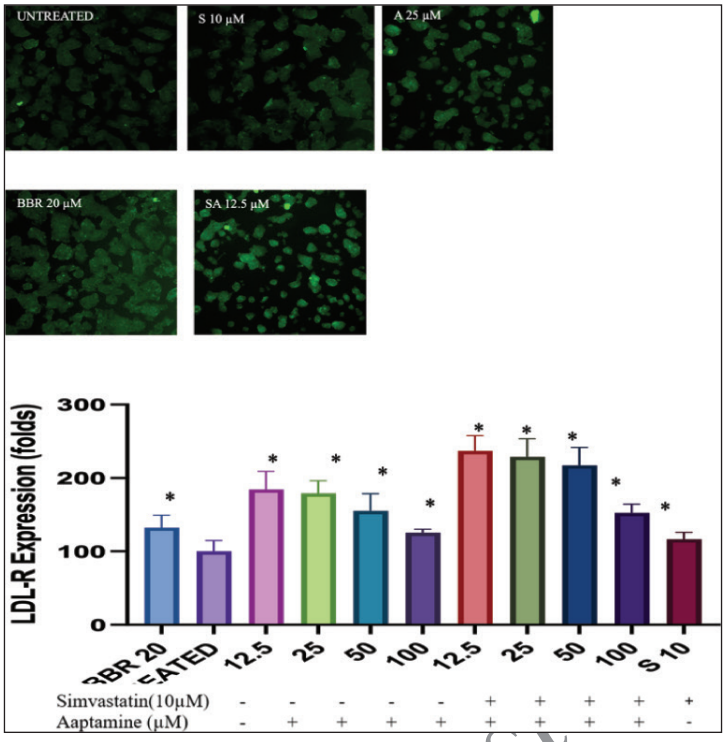


**Figure 6.** The presence of LDLR on the cell surface of liver cells after being treated either with aaptamine (A) or statin (S) or a combination of aaptamine and statin (SA) for 24 hours. HepG2 cells were also treated with 20 µM of berberine sulphate (BBR) as positive control and 1% (v/v) DMSO as untreated control for 24 hours. After treatment, the cells were immune stained with rabbit anti-LDLR primary antibody for 1 hour. Cells were subsequently incubated in the dark for 1 hour with DyLight™ 488-Conjugated secondary antibody. The intensity of FITC-stained fluorescence was observed using a Nikon Eclipse Ti2-E fluorescence microscope (20X magnification). Quantitative analysis of the of aaptamine and simvastatin on LDL-R levels. Fluorescence intensity was analysed and measured with ImageJ.



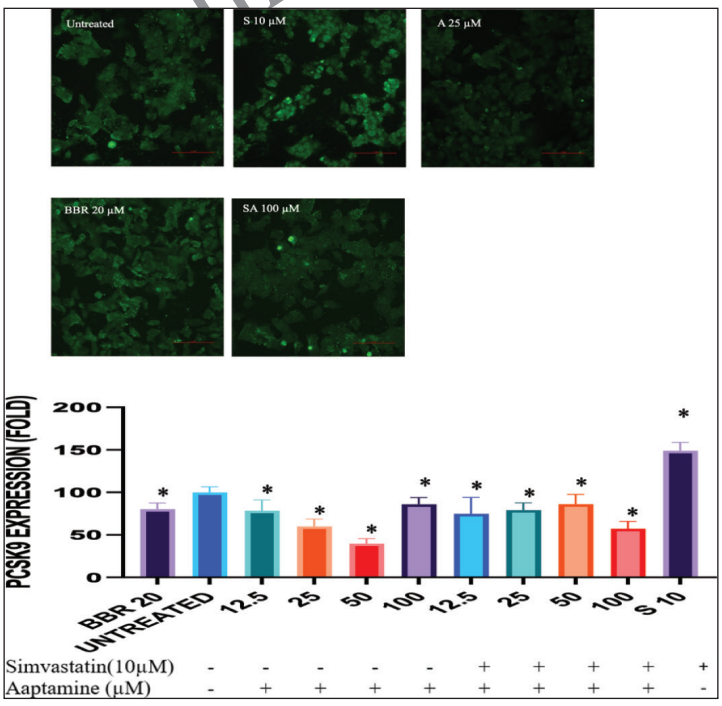


**Figure 7.** The expression of PCSK9 on the cell surface of liver cells after being treated either with a single treatment of aaptamine or statin or a combination of aaptamine and statin for 24 hours. HepG2 cells were also treated with 20 µM of berberine sulfate as positive control and 1% DMSO as untreated control for 24 hours. After treatment, the cells were immune stained with rabbit anti-PCSK9 primary antibody for 1 hour. Cells were subsequently incubated in the dark for 1 hour with DyLight™ 488-Conjugated secondary antibody. The intensity of FITC-stained fluorescence was observed using a Nikon Eclipse Ti2-E fluorescence microscope (20X magnification). Fluorescence intensity was analysed and measured with ImageJ.

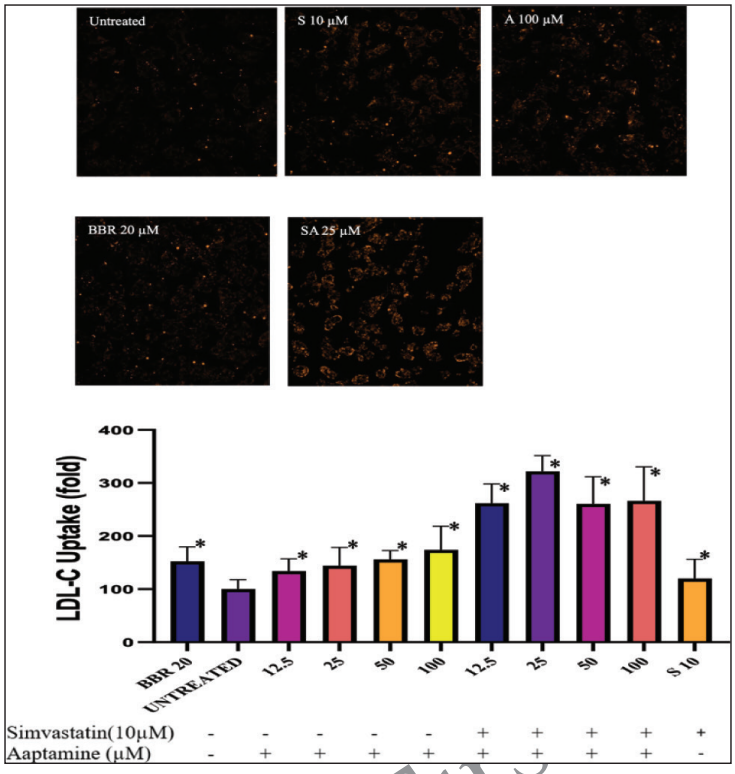


**Figure 8.** The rate of LDL-C uptake when HepG2 cells were treated either with individual aaptamine or statin or a combination of aaptamine and statin for 24 hours. HepG2 cells were also treated with 20 µM of berberine sulfate as positive control and 1% DMSO as untreated control for 24 hours. At the end of the treatment, the culture medium was replaced with 100 µl/well LDL-DyLight™ 550 working solution and incubated for an additional 3–4 hours. Images were captured using a Nikon Eclipse Ti2-E fluorescence microscope, which measured excitation and emission wavelengths 540 and 570 nm, respectively (20X magnification). Fluorescence intensity was analysed and measured with ImageJ.



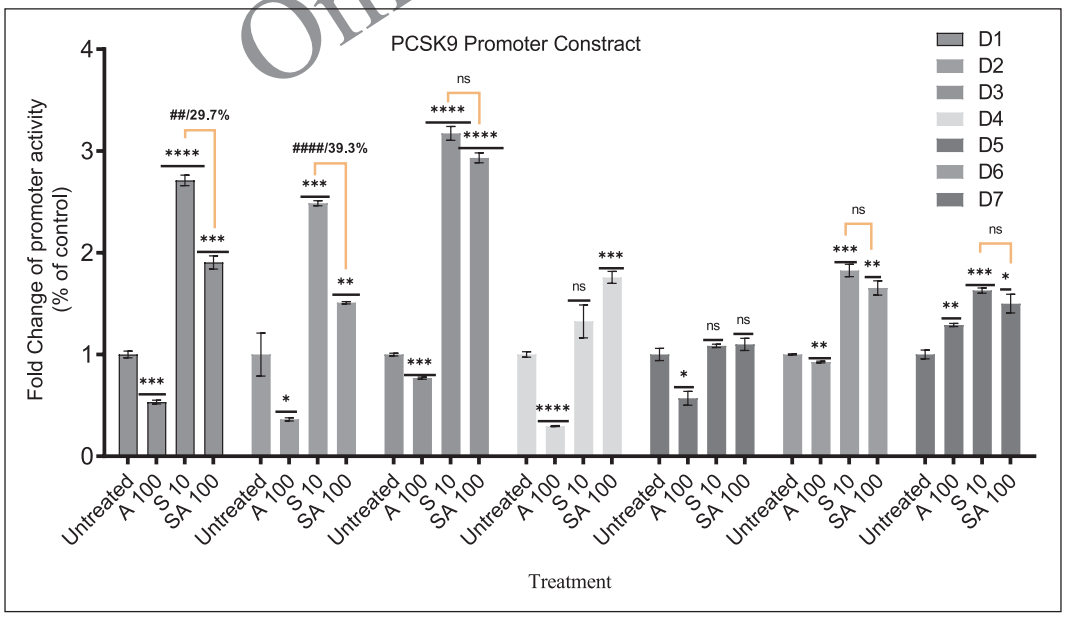


**Figure 9.** Identification of CAEs involved in aaptamine-inhibitory action on statin in regulating PCSK9 promoter activity. Seven 5' end deletion PCDK9 promoter-reporter constructs (D1-D7) were transiently transfected individually into HepG2 cells and treated with either 100 µM aaptamine (A) or 10 µM simvastatin (S) or a combination of simvastatin and aaptamine (SA) or 1% (v/v) DMSO as negative control (untreated). Following treatment, cell extracts were prepared and subjected to dual-glo luciferase assay. The data shown are from three independent experiments, and each bar represents the mean ± SD of triplicate samples. Statistical analysis comparing treated cells with untreated control was performed by unpaired *t*-test with significant value *$p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, and **** $p < 0.0001$. Statistical analysis comparing simvastatin treated cells with co-treatment of simvastatin and aaptamine was carried out by unpaired *t*-test with significant value ## $p < 0.01$; #### $p < 0.0001$, and ns, non-significant.

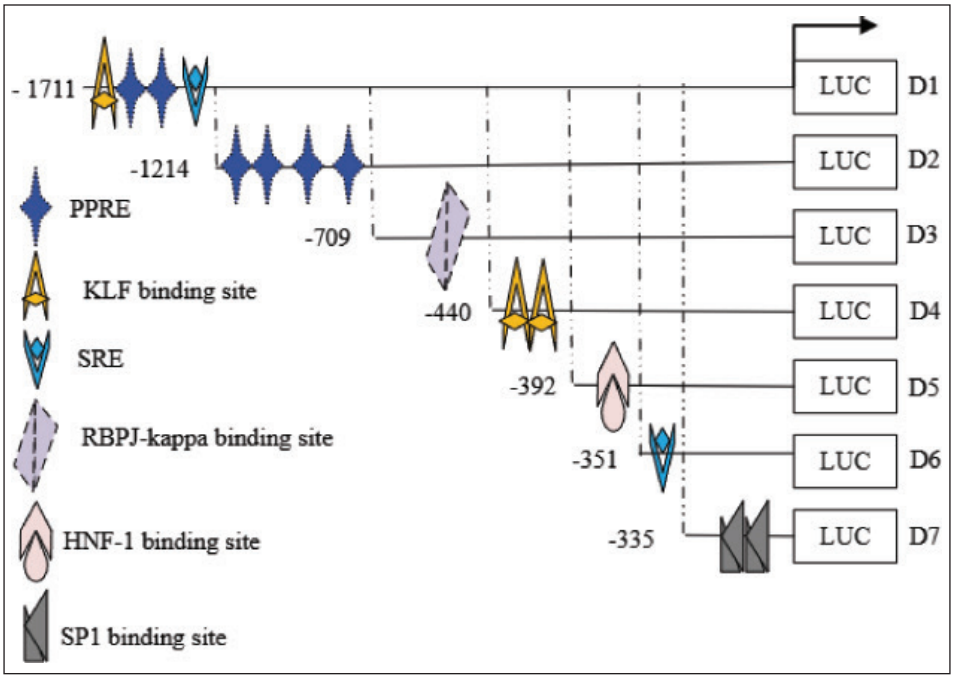


**Figure 10.** Schematic representation of the predicted distribution of transcription factor binding sites on the PCSK9 promoter. The arrow indicates the transcriptional start site (TSS). The MatInspector software was utilised to predict transcription factor binding sites on the PCSK9 promoter. PPRE: peroxisome proliferator response element; KLF: Krüppel like factor; SRE: sterol regulatory element; RBPJ-kappa: recombination signal binding protein for immunoglobin kappa J; HNF-1: hepatocyte nuclear factor 1; SP1: specificity protein 1.



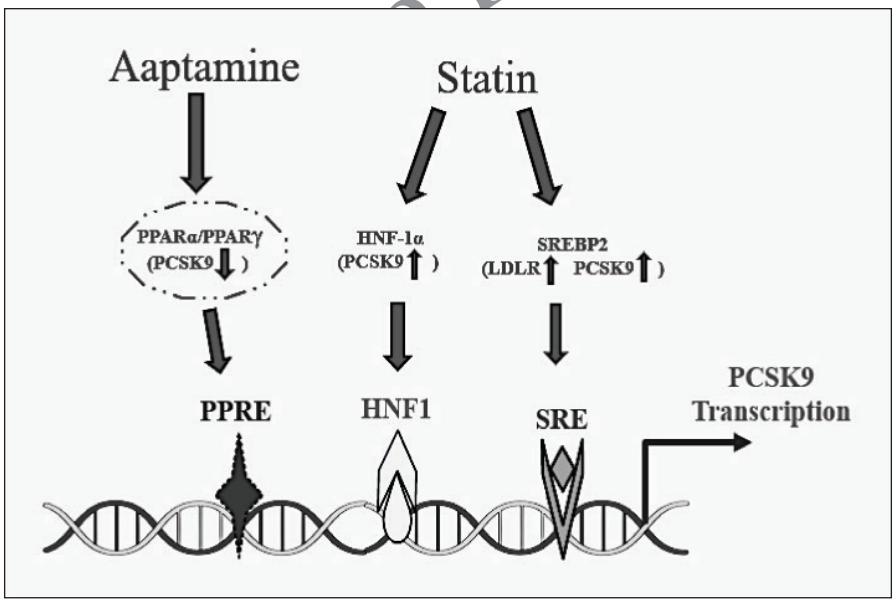


**Figure 11.** Schematic diagram of proposed mechanism of aaptamine action. Statin treatment elevates the expression of transcription factors SREBP-2 and HNF1-α. Since binding sites for these transcription factors are present in both the PCSK9 and LDLR promoters, statins increase the transcription of both genes, leading to enhanced PCSK9-mediated degradation of LDLR. However, we proposed that the binding site PPRE on PCSK9 promoter by activating PPARα or PPARγ mediates the effects of aaptamine on statin in reducing PCSK9 transcriptional activity.

significantly increased the promoter activity of the D7 fragment by 29% as compared to the control. As for simvastatin, the drug significantly upregulated the transcriptional activity of D1, D2, D3, D6, and D7 promoter fragments to 171.1%, 148.7%, 217.2%, 82.5%, and 62.8% of control levels, respectively. Interestingly, co-treatment of both mediators significantly reduced simvastatin-induced transcriptional activity of D1 and D2 promoter fragments by 29.7% and 39.3%, respectively.

Taken all together, the results above, that CAEs found in D1 and D2 fragments, but were absent in D3, D4, D5, D6, and

D7, were responsible for mediating the aaptamine inhibitory action on statin in regulating PCSK9 promoter activity.

### 3.6. Identification of the potential transcription factor binding sites (TFBSs) on the PCSK9 promoter

The analysis of potential TFBS in the PCSK9 promoter was conducted using MatInspector software (Fig. 10). TFBS analysis revealed the presence of four predicted sites between the region −1,711/−1,214 of the D1 promoter fragment. Two PPREs located between the positions −1,521/−1,499, and −1,506/−1,484, a Krüppel like factor (KLF) binding site was identified upstream of the PPRE binding site located between regions −1,629/−1,611 and a sterol regulatory element (SRE) at the location −1,237/−1,223.

The deletion of 497 bp from the 5' proximal region of D1 to generate the D2 fragment identified four additional PPREs located between the regions −1,200/−1,178, −1116/-1,094, −746/−724, and −739/−717, respectively, at the 5'end of the fragment. No similar binding sites were identified in the D3, D4, D5, D6, and D7 fragments (Fig. 10). The analysis of the region between −709/−440 of the 5' end of the D3 fragment identified the presence of the recombination signal binding protein for immunoglobulin kappa J region (RBPJ – kappa) binding site located between regions −549/−537.

Sequential deletion analysis of the 269 bp region at the 5' end from D3 to generate the D4 promoter fragment revealed additional KLF binding sites located between regions −434/−416 and −430/−412, respectively. The removal of a 48 bp segment from D4 to generate the D5 fragment identified the binding site for hepatocyte nuclear factor 1 (HNF1) within the region −387/−371. The predicted sterol regulatory element binding proteins binding sites, SRE was identified at the 5' end of the D6 promoter fragment at positions −346/−337. Additional transcription factor binding sites, specifically SP1 (−192/−176 and −197/−179), were identified within the D7 fragment. Figure 11 shows the Schematic diagram of proposed mechanism of aaptamine action. We proposed that the binding site PPRE on PCSK9 promoter by activating PPARα or PPARγ mediates the effects of aaptamine on statins in reducing PCSK9 transcriptional activity.

## 4. DISCUSSION

Atherosclerosis, a major contributor to cardiovascular diseases, remains the primary cause of mortality in Malaysia and worldwide [33]. The pathophysiological condition results from increased levels of oxidised LDL-C in the bloodstream, causing lipid accumulation in the inner lining of blood vessels and the formation of atherosclerotic plaque [8]. PCSK9 plays a significant role in the regulation of plasma LDL-C levels by binding to the extracellular domain of the hepatic LDLR. The formation of the PCSK9-LDLR complex leads to the intracellular degradation of LDLR in lysosomes, thereby reducing the amount of LDLR in hepatocytes, which in turn increases plasma LDL-C levels [17,30,34,35].

Gain-of-function mutations of PCSK9 in humans increase the binding affinity of the enzyme to LDLR, thereby enhancing the degradation of LDLR [36,37]. In contrast, loss-of-function mutations in PCSK9 result in lower LDL-C levels, thereby diminishing cardiovascular risk [38]. LDLR and PCSK9 share a common regulatory pathway. The transcription of both genes is induced by a decrease in cellular cholesterol levels via the sterol regulatory element-binding protein (SREBP) [39]. Statins are effective medications for lowering cholesterol, as they inhibit the overexpression of SREBP2, thereby increasing the transcription of LDLR. SREBP-2 specifically activates genes involved in cholesterol biosynthesis [40,41]. The identification of the SRE-1 motif within the promoter regions of PCSK9 and LDLR, along with the augmented binding of SREBP-2 to SRE, suggests that statin-induced overexpression of SREBP2 promotes the transcriptional activity of PCSK9 and LDLR [42–44]. Elevated PCSK9 transcription specifically reduces the efficacy of statins in lowering LDL cholesterol [18].

Aaptamine was shown to reduce PCSK9 gene expression through PPRE, rather than SRE (unpublished results). Aaptamines represent a distinct class of marine alkaloids that have garnered significant interest from researchers across various fields, owing to their diverse biological activities and unique structural characteristics [45]. Previous studies indicate that aaptamine increases the transcriptional activity of the PPRE located in the promoters of target genes [20,21,46]. PPREs were identified in the promoter of PCSK9. It is essential to examine the role of aaptamine in mitigating the effects of statins on the regulation of PCSK9 expression and in promoting the expression of LDLR and LDL-C uptake by liver cells.

Aaptamine significantly reduced the mRNA expression levels of PCSK9 across all concentrations tested. In contrast, statins significantly increase PCSK9 mRNA levels, which are attenuated when aaptamine is co-introduced into the cells. Wu and colleagues examined the effects of PCSK9 inhibition through Diallyl disulphide (DADS) derived from garlic [47]. HepG2 cells were cultured with LPS at a concentration of 1,000 ng/ml, with or without the addition of DADS at 80 mg/ml and atorvastatin at 5 mmol/l. Atorvastatin produced a greater increase in PCSK9 expression compared to the control group. The co-administration of atorvastatin and DADS diminished the induction of PCSK9 elicited by the statin. Wu *et al.* [47] demonstrated that DADS inhibits statin-induced PCSK9 expression and may enhance the efficacy of statin therapy. Dong *et al*. [48] demonstrated that the compound silibinin A (SIL), acknowledged as a traditional Chinese medicine, inhibits PCSK9 promoter activity. This study examined the effect of silibinin A on the expression levels of PCSK9 mRNA in HepG2 cells subjected to high concentrations of silibinin A. The results indicated that silibinin A decreased PCSK9 mRNA levels in a dose-dependent manner. Silibinin A, at a concentration of 100 μM, decreased PCSK9 mRNA levels by 65.8% [48].

Statins significantly increase the level of cell surface PCSK9 [48–50]. Dong *et al*. [48] found that PCSK9 mRNA expression levels increased 2.3-fold following treatment of HepG2 cells with 10 μM atorvastatin. Co-incubation of HepG2 cells with atorvastatin and silibinin A for 12 hours resulted in a 78.1% decrease in the atorvastatin-induced increase of PCSK9 mRNA levels [48]. This study indicates that treatment with statin significantly increased the mRNA expression of PCSK9 in cells, reaching nearly three times that of the untreated control. Kim *et al*. [50] investigated the impact of piceatannol administered

alongside rosuvastatin or simvastatin. Piceatannol therapy significantly reduced statin-induced PCSK9 mRNA expression [50]. Statins reduce cholesterol synthesis by inhibiting HMG-CoA reductase [10]. Decreased intracellular cholesterol levels activate SREBP2, leading to its translocation from the endoplasmic reticulum (ER) to the Golgi complex, where it is cleaved to produce mature SREBP2 [51]. The mature SREBP2 translocates to the nucleus, enhancing its binding to SRE and subsequently inducing PCSK9 gene expression [11,27]. PCSK9 promoters contain two conserved motifs essential for cholesterol regulation: a SRE and a Sp1 site [39].

While SRE is present in LDLR promoters, elevated levels of PCSK9 restrict the increase in hepatic LDLR protein levels induced by statins, potentially limiting the therapeutic efficacy of statins in reducing circulating LDL-C [52]. When cells were cotreated with simvastatin and aaptamine at concentrations ranging from 12.5 to 100 µM, the mRNA expression of PCSK9 was reduced compared to the untreated control. These results were corroborated by Mohamad *et al.* [22], who found that aaptamine decreased PCSK9 promoter activity in a dose-dependent manner. PCSK9 inhibitors, when used in conjunction with a statin, result in a 40%–72% decrease in LDL-C levels [53]. The findings indicate that aaptamine may serve as a PCSK9 inhibitor, essential for maintaining or increasing the levels of LDLR on the cell surface to facilitate LDL-C uptake.

Research indicates that fenofibrate, a ligand for PPARα, decreases the levels of PCSK9 mRNA and protein in the liver of wild-type mice [54]. Moreover, research indicates that PPARα activation suppresses statin-induced PCSK9 expression by inhibiting PCSK9 promoter activity [55]. Fractions obtained from *Acaudina molpadioides* were found to reduce the activity of the promoter and the expression of PCSK9 mRNA [24]. N-(2,3-dihydro-1H-inden-2-yl)-2-methoxybenzamide, a compound derived from methylbenzoate present in *Acanthaster planci*, demonstrated the ability to inhibit the transcriptional activity of PCSK9 in HepG2 cells [56]. Yang *et al.* [57] demonstrated that fucoidan, sourced from the brown seaweed *Ascophyllum nodosum*, significantly increases PPARα expression in the liver, thereby promoting lipid transfer from plasma to the liver through the activation of SR-B1 and LDLR, while inhibiting PCSK9. Furthermore, it enhances lipid metabolism through the activation of PPARα, LXRβ, ABC transporters, and CYP7A1 [57].

Our study found that statin treatment reduced premature PCSK9 protein expression while increasing mature PCSK9 protein expression by 4-fold. Co-treatment with simvastatin and aaptamine resulted in a significant downregulation of both premature and mature PCSK9 protein expressions (Fig. 5). A single treatment with aaptamine resulted in a significant downregulation of protein expression for both premature and mature PCSK9 (Fig. 4) when compared to the untreated control. PCSK9 undergoes autocatalytic cleavage during maturation in the ER. Mature PCSK9 is synthesised in the liver and subsequently released into the bloodstream [58].

Hwang *et al*. [59] demonstrated that lipoprotein depletion led to an increase in the expression of both precursor and mature PCSK9 proteins. Treatment with butein at concentrations beginning at 10 µM resulted in a significant and dose-dependent reduction in both precursor and mature PCSK9 protein expression [59]. Dong *et al*. [48] evaluated the effect of 100 µM silibinin A on PCSK9 expression in HepG2 cells across different time intervals. The treatment of HepG2 cells with silibinin A resulted in a significant reduction in PCSK9 protein expression levels over time [48]. Hwang *et al*. [59] investigated the capacity of butein to modulate the expression levels of LDLR and PCSK9 in HepG2 cells, both independently and synergistically. HepG2 cells underwent treatment with or without a statin and butein in delipidated serum conditions. Statin treatment led to an increase in the expression of LDLR and PCSK9 proteins; in contrast, butein caused a modest increase in mature LDLR protein while significantly decreasing PCSK9 protein levels [59].

Chen *et al*. [32] investigated the impact of tanshinone IIA in conjunction with statins on PCSK9 expression and LDL uptake in HepG2 cells. Treatment with lovastatin or simvastatin alone led to a significant elevation in PCSK9 mRNA and mature protein expression. According to Dong *et al*. [60], western blot analysis of HepG2 cell lysates indicated that atorvastatin increased PCSK9 protein expression levels by 51.0%, whereas silibinin A reduced this effect by 43.2% [48]. Li and colleagues [60] examined the effect of Berberine on PCSK9. The mRNA expression was evaluated by treating cells with 1 µM of fluvastatin, lovastatin, or simvastatin, in both the absence and presence of two different doses of Berberine. Statins increased PCSK9 mRNA levels to more than double that of the control; however, this effect was not observed in cells treated concurrently with Berberine at concentrations of 13 µM and 26 µM [60]. CM3-SII, a novel alkali-extracted polysaccharide, was obtained from the fruiting body of *Cordyceps militaris* by Wang *et al*. [61]. The CM3-SII intervention significantly decreased PCSK9 secretion by about 58% ($p < 0.01$) at a concentration of 200 µg/ml when compared to the vehicle or simvastatin therapy [61].

Figure 8 illustrates that an increase in cell surface LDLR correlates with enhanced LDLC uptake by liver cells when co-treated with aaptamine and statin. Several other compounds exhibited increased LDLC uptake by upregulating LDLR and downregulating PCSK9. Chae and colleagues found that exposure of HepG2 cells to sauchinone resulted in elevated levels of LDLR mRNA, enhanced synthesis of LDL-receptor protein, and increased uptake of LDLC into the cells [62]. Jeong and collegues [27] demonstrated that allicin and capsaicin have hypolipidemic effects by enhancing LDLR expression through SREBP2 activation and reducing PCSK9 expression via HNF1α suppression. The levels of secreted PCSK9 in the culture medium were reduced, which may enhance LDL-C uptake in HepG2 cells [27].

Hwang *et al*. [59] found that treatment with butein alone led to minor increases in LDLR expression. However, the combination of statin and butein markedly enhanced LDLR protein expression, suggesting a potential synergistic effect between these therapies. The elevation of PCSK9 caused by statins was reduced with concurrent treatment of butein, due to the PCSK9-inhibitory effects of butein [59]. Chen et al. [32] demonstrated that co-treatment with tanshinone IIA significantly reduced the expression of PCSK9 induced by lovastatin or

simvastatin. The co-treatment of cells with tanshinone IIA and two statins significantly enhanced LDL uptake activity compared to treatment with lovastatin or simvastatin alone [32]. They demonstrated that the combination of tanshinone IIA and statins significantly decreased statin-induced PCSK9 gene expression while enhancing LDLR activity in HepG2 cells [32]. Kim *et al*. [63] demonstrated that statins, specifically rosuvastatin or simvastatin, increased the expression of PCSK9 and LDLR proteins and mRNA in the presence of mevalonate. Following treatment with a combination of each statin and piceatannol, LDLR exhibited significant stabilisation, while PCSK9 degradation occurred in a concentration-dependent manner concerning piceatannol [63]. The elevation of PCSK9 levels resulted in an increased degradation rate of the LDLR protein. Certain statins are recognised for increasing plasma PCSK9 levels, which partially diminishes the effect of statins on LDLR expression [62].

Statins reduce LDL-C levels through an additional mechanism by increasing LDLR levels via the SREBP-2 pathway, thereby further decreasing circulating LDL-C. PCSK9, which degrades LDLR, is upregulated by statins in both *in vivo* and *ex vivo* conditions. Mayne *et al*. [44] showed that simvastatin significantly upregulated LDLR protein expression in the HepG2 cell line at a concentration of 1 μM. LDLR expression exhibited a greater upregulation compared to PCSK9, with ratios of 2.0× versus 1.5× at 1 μM and 2.6× versus 1.5× at 10 μM simvastatin, respectively (44). This suggests that although PCSK9 functions as a negative regulator of LDLR and is upregulated with LDLR through the SREBP-2 pathway when statins are present, the increase in PCSK9 at the protein level does not fully negate the upregulation of LDLR [44].

The PCSK9 promoter, spanning from −1711 to −94, represents the primary functional region that encompasses multiple CAEs essential for the regulation of the PCSK9 gene [27]. Analysis of transient transfection involving 5' end deletion of PCSK9 promoter fragments indicates that transcriptional control at the promoter level significantly regulates not only the inducible effects of statin but also inhibitory effects of aaptamine on statin (Fig. 9). The findings aligned with previous studies indicating that various small molecules influence the gene expression of PCSK9 through the transcriptional activity of its promoter. The plant-derived hypocholesterolemic compound berberine (BBR) increased LDLR expression and decreased PCSK9 levels by coordinating the reduction of HNF-1α and SREBP-2 [49]. Silibinin A, a compound derived from traditional Chinese medicine, was shown to reduce the expression levels of PCSK9 in HepG2 cells by diminishing the activity of the PCSK9 promoter in a dose- and time-dependent manner [64]. Icaritin, derived from *Capsella bursa-pastoris*, markedly reduced PCSK9 secretion through the inhibition of SREBP-2 and HNF-1α [65]. Qiao *et al*. [66] investigated the regulatory mechanism of 3 seconds, a analogues of effective small-molecule PCSK9 inhibitor, 7030B-C5 suggesting it may target the transcription factor HNF1α and/or HINFP upstream of PCSK9 transcription [66].

Investigations of the ~ 650 bp proximal region of the PCSK9 promoter have identified three promoter elements that influence PCSK9 expression. A Sp1 site located furthest upstream was found to have a modest role in the transcription of PCSK9 [27]. A SRE serves as the binding site for sterol-regulatory element binding proteins and is located ~330 bp upstream of the translation start site, playing a crucial role as a *cis*-element in the regulation of PCSK9 transcription [51,67,68]. Another nuclear factor involved in PCSK9 expression is HNF1α (hepatocyte nuclear factor 1 homeobox A), which is also located upstream of the SRE motif. It exists in non-human primates and rodents. It is interesting to note that based on MatInspector analysis of PCSK9 promoter in our study, it was revealed that similar CAEs were also present. Based on 5'end deletion, D2 fragments produced the highest reduction in the transcriptional activity when the cells were treated with a combination of aaptamine and simvastatin, as compared to simvastatin alone. MatInspector analysis identified four PPRE at the 5' end of the D2 fragment (−1214/−709). The existence of PPRE may elucidate its function in moderating the inhibitory effect of aaptamine on the action of statin on the promoter activity of D1 and D2 fragments.

However, this study did not directly assess the activation of PPARα or PPARγ, nor did it employ PPRE-specific luciferase reporter assays to confirm their functional involvement. A more comprehensive investigation into the underlying molecular mechanisms, including transcription factors and signalling pathways such as PPARα and PI3K, MEK1/2, and PKC, could provide significant insights into its regulatory role of aaptamine in PCSK9 expression. Unlike FDA-approved PCSK9 monoclonal antibodies (such as alirocumab and evolocumab), which bind directly to circulating PCSK9 to prevent LDLR degradation, aaptamine acts at the transcriptional level, modulating gene expression. This study demonstrated that aaptamine significantly inhibited PCSK9 mRNA and protein expression in a dose-dependent manner, thereby indirectly increasing LDLR levels and LDL-C uptake.

From a clinical standpoint, the prospective utility of aaptamine resides in its capacity to disrupt the statin-induced PCSK9 feedback loop, which otherwise limits statin efficacy by reducing LDLR availability. As a marine-derived small molecule, aaptamine may function as a novel oral small-molecule adjunct to statins, particularly for patients who exhibit statin resistance or intolerance. However, before clinical translation can be considered, extensive pharmacokinetic, toxicological, and *in vivo* efficacy investigations in animal models, and ultimately in human subjects, are essential. These steps are very important for figuring out possible off-target effects, the best dose, and the safety profile.

## 5. CONCLUSION

The findings demonstrate that aaptamine reduced the inducible effect of statin on PCSK9 expression, thereby increasing the level of cell surface LDLR and enhancing LDL-C uptake, which may subsequently decrease LDL-C levels in the bloodstream. Marine resources functioning as PCSK9 inhibitors may represent promising novel therapeutic agents, either alone or in combination with statins, to reduce circulating

LDL-C levels and potentially mitigate the progression of atherosclerosis.

Aaptamine may possess therapeutic promise for metabolic diseases, including non-alcoholic fatty liver disease and metabolic syndrome, owing to its documented antioxidant and anti-inflammatory characteristics, in addition to its involvement in cardiovascular regulation. Given the overlap in molecular pathways, future studies should explore its broader application in cardiometabolic health.

This research, like all research studies, possesses inherent limitations. The study utilized an *in vitro* model, which, although beneficial for certain experiments, has its own limitations to entirely mimic the complex nature of human physiology.

Future research should assess the effects of aaptamine on lipid metabolism in whole-body systems, particularly focusing on its influence on LDL-C clearance and the advancement of atherosclerosis in animal models. Clinical trials are essential to validate the efficacy and safety of this treatment in individuals, particularly those with hypercholesterolaemia or statin intolerance.

## 6. ACKNOWLEDGMENT

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## 7. AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

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## 9. CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

## 10. ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

## 11. DATA AVAILABILITY

All data generated and analyzed are included in this research article.

## 12. PUBLISHER'S NOTE

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## 13. USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

The authors declares that they have not used artificial intelligence (AI)-tools for writing and editing of the manuscript, and no images were manipulated using AI.

## 14. INFORMED CONSENT

All authors have consented to submit an article.

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