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Research Article

The Association between Genetic Variants and Gene Expression in RAAS Genes Using Captive-Bred Vervet Monkeys (*Chlorocebus aethiops*)

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Mendelian genetics contribute largely to the development of hypertension; therefore, the identification of genetic variants related to blood pressure (BP) regulation remains crucial and may reveal new therapeutic drug targets. The purpose of the present study was to screen the captive-bred Vervet colony for salt-sensitive sequence variants or single nucleotide polymorphisms (SNPs) in the selected Renin-Angiotensin-Aldosterone System (RAAS) genes associated with salt sensitivity. Blood samples were collected from 16 captive-bred Vervet monkeys for genotyping and gene expression analysis. The impact of the identified sequence variants was determined using online prediction tools. Sanger sequencing analysis revealed 21 sequence variants in *AGT*, *CYP3A5*, *GRK4*, and *SCL4A5*, of which 19 were novel and two were previously reported in humans. All novel variants were either predicted to be polymorphic, disease-causing, or possibly damaging by prediction tools. Furthermore, the mRNA expression for *AGT* was significantly higher in the normal BP group (p value = 0.02), and a similar trend was observed for *CYP3A5* and *GRK4*, whereas *SCL4A5* was higher in the hypertensive group. The identified salt-sensitive variants specifically in *GRK4* may be suggestive to be the attributing factor of the elevated BP levels in these captive-bred Vervet monkeys. Therefore, RAAS variants could be considered as a biomarker to identify the potential risk of developing hypertension in both humans and nonhuman primates.

1. Introduction

Hypertension is a public health concern and can lead to cardiovascular, cerebrovascular, and kidney diseases [1]. The prevalence of hypertension varies with age, sex, and ethnicity. Currently, the population data indicates that the incidence of hypertension ranges from 6.6 to 26 million in developed countries while in developing countries it is 639 million, and it is projected that by 2025, the number of patients with hypertension would be 1.56 billion globally [2, 3]. Excess dietary salt intake has been reported to predominantly contribute to hypertension [4], and this is associated with increased cardiovascular events and mortality irrespective of basal blood pressure (BP) levels [5]. In animal studies, salt-resistant rats do not develop an elevated BP after being fed high-salt diet (8% NaCl), while sensitive rats show elevated BP [6]. This means that a high-sodium diet is deleterious while a low-sodium diet is regarded as part of a healthy lifestyle and treatment of hypertension [7]. Although salt sensitivity, hypertension, and related cardiovascular disease (CVDs) result from the interaction of genes with environmental factors such as stress and diet [8], the underlying mechanism of salt sensitivity is not well understood [9]. Several studies have proved that Mendelian genetics contribute largely to the development of hypertension. Evidence from previous reviews estimated that genes contribute 30–50% to the pathogenesis of BP [10, 11]. Therefore, the identification of genetic variants related to BP regulation remains crucial and may reveal new therapeutic drug targets.

Several Renin-Angiotensin-Aldosterone System (RAAS) genes have been suggested to play a vital role in BP response to salt [12]. These genes include the sodium bicarbonate cotransporter gene (*SLC4A5*), G protein-coupled receptor

kinase 4 (*GRK4*), cytochrome P450 3A5 (*CYP3A5*), and angiotensinogen (*AGT*). *SLC4A5* is a transmembrane protein that functions as an electrogenic cotransporter of bicarbonate and sodium [13], and it is known to be associated with hypertension of which five polymorphisms have been reported [14, 15]. The *GRK4* gene regulates dopamine receptors, which are important in regulating sodium transport and BP [16]. In hypertensive patients, three polymorphisms (R65L, A142V, and A486V) have been reported to show an increase in *GRK4* activity in the renal tubule and cause phosphorylation and agonist-independent uncoupling of dopamine 1 receptor (D1R) [17, 18]. Additionally, overexpression of *GRK4* causing hypertension has been observed in transgenic mice that were fed high-salt diet (A486V) and on a regular diet (A142V) [19, 20]. In South Africa, *GRK4* polymorphisms are more common in the African black population and are associated with impaired sodium excretion [21]. Moreover, the *CYP3A5* gene has been reported to play a role in sodium reabsorption and BP regulation in the presence of a premature stop codon (rs776746) that influences salt and water retention and reduces its expression [22]. *AGT* is among the components that are involved in the activation cascade of the RAAS, which act together to regulate BP by maintaining vascular tone and the balance of water and sodium [23].

In this study, four RAAS genes (*AGT*, *CYP3A5*, *GRK4*, and *SLC4A5*) were screened for sequence variants followed by gene expression analysis to investigate the association between salt sensitivity and hypertension development using the captive-bred Vervet monkey model. To date, several studies have demonstrated nonhuman primates (NHPs) to be effective research models to evaluate the diseases afflicting humans. NHP is also known to be an excellent animal model for various noncommunicable diseases (NCDs) and shares various characteristics with humans [24]. These include the sodium-lithium counter transport activity (SLC) in their red blood cells which can lead to salt sensitivity [25]. Therefore, the use of the Vervet model is proposed in this study as a model of choice to better understand the genetic contribution of RAAS genes and find new therapeutic approaches to combat hypertension.

2. Materials and Methods

2.1. Animal Selection. The ethical clearance was obtained from the Ethics Committee for Research on Animals at the South African Medical Research Council (SAMRC) (ECRA; Ref 10/18). Sixteen captive-bred Vervet monkeys were selected based on age, gender, BP, and lipogram parameters (Tables 1, S1). Sample collection procedure and housing conditions were according to the Primate Unit Delft Animal Center (SAMRC/PUDAC) standard operating procedures and the revised South African National Standard for the Care and Use of Animals for Scientific Purposes (South African Bureau of Standards, SANS 10386, 2008).

The pedigree demonstrated two families with half-siblings (Figure S1), in which four animals were from the same family tree, consisting of three half-siblings with normal BP and one half-brother who was hypertensive (family A). Family B had two half-sisters, one with normal BP and another one was hypertensive while the rest of the animals were from independent families (family C, D, E, F, G, and H). Additionally, three animals were wild caught from Modderfontein farm, Potchefstroom, in South Africa before being housed at PUDAC in 2012 and two of these animals were infertile.

2.2. Clinical Assessment, DNA Extraction, and Quantification. Animals were handled after chemical restraint with Ketamine (Kyron Laboratories, South Africa) at 10 mg/kg. Once an animal was fully unconscious, phenotype traits such as BP, peripheral capillary oxygen saturation percentage (SpO2 %), body weight, and heart rate were measured. Thereafter, blood (2–4 mL) was collected via femoral venipuncture into 4 mL Vacutainer® EDTA Tubes for genomic DNA extraction using a NucleoSpin Genomic Blood DNA purification kit (Macherey-Nagel, Germany). The concentration, quantity, and purity of DNA were immediately measured using NanoDrop 2000 spectrophotometer analysis (Vacutec, South Africa), and DNA quality was confirmed by standardizing 2% gel electrophoresis that was stained with 2 μL of ethidium bromide (EtBr).

2.3. Candidate Gene Selection and Sequence Retrieval for Genotyping. Bioinformatics research tools such as the NCBI GENBANK, Ensembl, and UCSC genomic browser were used to retrieve the genomic sequences for the selected genes (*AGT*, *CYP3A5*, *GRK4*, and *SLC4A5*) using the Green monkey (*Chlorocebus sabaeus*) as a reference sequence (Table S2). Furthermore, NCBI primer BLAST and PrimerQuest Tool (Whitehead Scientific, South Africa) were employed to design primers targeting the coding exons for all the selected genes (Table S3).

2.4. PCR Amplification and DNA Sequencing. Selected genes were amplified by PCR using the Veriti™ 96-Well Thermal Cycler (Applied Biosystems®, USA). Each standard PCR reaction (25 μL) consisted of the following reagents: GoTaq PCR Master Mix (2x) (Promega, USA), 2 mmol/L forward and reverse primer, 50 ng μL^{-1} DNA, and nuclease-free water. The cycling program was similar to the one that has been previously published [26]. Briefly, cycling conditions included denaturation at 94°C for 5 minutes, followed by 30 cycles at 94°C for 30 seconds, varying annealing (40–70°C) for 30 seconds (Table S3), and extension of 72°C for 1 minute, followed by a final extension of 72°C for 5 minutes. The PCR products were purified using the Wizard SV gel and PCR clean-up kit (Promega, USA). Based on the gel electrophoresis results, purified PCR products were sequenced and analyzed using ClustalW and ExPASy translate tool. The impact of the identified sequence variants was evaluated using online tools such as Polymorphism Phenotyping (PolyPhen-2) [27] and Sorting Intolerant from Tolerant (SIFT) website which is provided by Pauline Ng that predict whether a change in amino acid affects protein function. The Ensembl transcript ID of each gene was selected in MutationTaster [28], Human Splice Site Finder (HSF) [29], and Variant Effect Predictor (VEP) [30] to identify the impact of each variant.

Table 1: Clinical parameters.

Parameters	Normal group	Hypertensive group	p values
Age	>3–10 years	>3–10 years	
Gender	4 M and 4 F	4 M and 4 F	
Systolic BP (mmHg)	103.2 ± 5.343	167.6 ± 10.53	<0.0001
Diastolic BP (mmHg)	46.31 ± 8.094	72.81 ± 8.52	0.04
Total cholesterol (mmol/L)	4.05 ± 0.2353	3.6 ± 0.2659	0.23
Triglycerides (mmol/L)	0.5125 ± 0.1015	0.4025 ± 0.05081	0.35
LDL cholesterol (mmol/L)	1.575 ± 0.1688	1.475 ± 0.2016	0.71
HDL cholesterol (mmol/L)	2.2 ± 0.1282	1.8 ± 0.1268	0.04

Values are mean ± standard deviation; $p < 0.05$ is considered significant. BP: blood pressure; LDL: low-density lipoprotein; HDL: high-density lipoprotein; M: males; F: females.

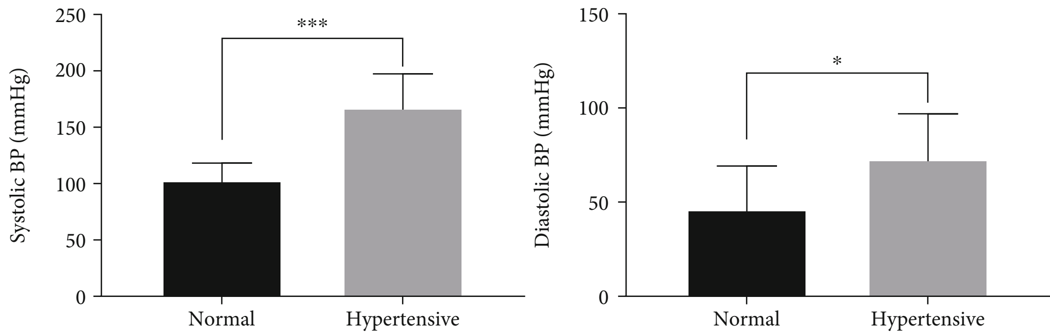

Figure 1: Captive-bred Vervet monkeys' systolic blood pressure (BP) and diastolic BP. The normal group had systolic BP less than 120 mmHg, while the hypertensive group had systolic BP above 130 mmHg. ***Significant difference ($p < 0.0001$) and *significant difference ($p < 0.05$).

Total RNA was also extracted from whole blood (2 mL) using a PAXgene blood RNA extraction kit according to the manufacturer's instructions (PreAnalytiX, Qiagen). The qPCR primer assays (Qiagen, Germany) designed for SYBR® Green-based RT-qPCR detection were used for relative gene expression of the selected genes. Selected primer assays were human-based (*Homo sapiens*) (Table S4), since the NHPs including Green monkey (*Chlorocebus sabaeus*) and Vervet monkey (*Chlorocebus aethiops*) assays are not yet available on the gene database. The PCR reaction consisted of 5 μL of 2X Power SYBR Green PCR Mastermix (Applied Biosystems, USA), 0.5 μL (1X) of 10X primer stock, 1 μL of cDNA, and 3.5 of water. Reactions were prepared in 96-well reaction plates. All RT-qPCR standards and cDNA samples were amplified in duplicates using the Applied Biosystems universal cycling conditions. A melt curve for secondary product detection was included in the RT-qPCR run. Data for relative expression was analyzed with the QuantStudio-3 Real-Time PCR System (Applied Biosystems, USA), and results were further analyzed using the delta-delta Ct method (2–ΔΔCt) [31]. The RT-qPCR data for each gene was normalized to the average of two housekeeping genes such as phosphoglycerate kinase 1 (*PGK1*: PPQ09326C) and glyceraldehyde 3-phosphate dehydrogenase (QT01192646).

2.5. Statistical Analysis. Data were expressed as means ± standard deviation between the groups. Statistical analysis was performed using GraphPad Prism, version 7.05. Statistical significance was calculated by using the Student t-test, and the statistically significant difference was set at $p < 0.05$.

3. Results

The selected animals showed no symptoms of being unwell or distressed. As indicated in Table 1, lipogram parameters for triglycerides and LDL cholesterol were not statistically different except for HDL-C ($p = 0.04$) which was significantly higher in the hypertensive group compared to the normal group. Phenotype traits such as body weight were also not statistically significant between the groups.

Systolic BP was significantly high in the hypertensive group compared to the normal group ($p < 0.0001$), and a similar trend was observed in diastolic BP ($p = 0.04$) (Figure 1).

Screening of RAAS genes such as *AGT*, *CYP3A5*, *GRK4*, and *SLC4A5* revealed 21 sequence variants in captive-bred Vervet monkeys (Table 2). The protein sequence alignment of the *AGT* gene showed that the identified sequence variants were located in a conserved region and predicted to be polymorphisms (V89A, P65P, T76T, and T318T) and

Table 2: Sequence variants identified in the colony of captive-bred Vervet monkeys (*Chlorocebus aethiops*).

Gene	Exon	Nucleotide change	Type of change	AA change	Type of mutation	MutationTaster	PolyPhen-2	SIFT	VEP
AGT	Exon 2	C195>T	Transition	P65P	Silent	Polymorphism	ND	Neutral	Silent variant
		A228>G	Transition	T76T	Silent	Polymorphism	ND	Neutral	Silent variant
		T266>C	Transition	V89A	Missense	Polymorphism	Benign	Neutral	Missense variant
	Exon 3	C954>A	Transversion	T318T	Silent	Polymorphism	ND	Neutral	Silent variant
	Exon 5	C1431>T	Transition	R477R	Silent	Disease-causing	ND	Neutral	Silent variant
CYP3A5	Exon 5	G373>C	Transversion	E125Q	SNPs	Polymorphism	PD	Neutral	Missense variant
	Exon 10	G885>T	Transversion	L295L	Silent	Disease-causing	ND	ND	Silent variant
		G888>A	Transition	V296V	Silent	ND	ND	ND	Silent variant
GRK4	Exon 3	G305>T	Transversion	L102R	SNP	Polymorphism	Benign	Neutral	Missense variant
	Exon 5	C533>T	Transition	A178V	SNP	Polymorphism	Benign	Neutral	Missense variant
	Exon 6	A587>G	Transition	Q196R	SNP	ND	Benign	ND	Missense variant
		G588>C	Transversion	Q196R	SNP	ND	Benign	ND	Missense variant
	Exon 11	G1241>A	Transition	S414N	SNP	Disease-causing	Benign	Deleterious	Missense variant
SLC4A5	Exon 6	A183>G	Transition	S61S	Silent	Polymorphism	ND	Neutral	Silent variant
	Exon 8	G540>T	Transversion	T180T	Silent	Disease-causing	ND	Neutral	Silent variant
	Exon 9	C705>T	Transition	R235R	Silent	Disease-causing		Neutral	Silent variant
	Exon 17	G1740>A	Transition	S580S	Silent	Disease-causing	ND	Neutral	Silent variant
	Exon 18	C>1944T	Transition	G648G	Silent	ND	ND	ND	Silent variant
		G1947>C	Transversion	L649F	SNPs	ND	Benign	Neutral	Modifier
	Exon 24	C2817>T	Transition	T939T	Silent	Disease-causing	ND	Neutral	Modifier
	Exon 25	G2892>A	Transition	V964V	Silent	ND	ND	Neutral	ND

AA: amino acids; PD: possibly damaging; ND: not determined; SNP: single nucleotide polymorphism; SIFT: Sorting Intolerant from Tolerant; VEP: Variant Effect Predictor.

disease-causing (R477R) when blasted on the MutationTaster tool (Table 2). Genotyping findings further showed that the selected captive-bred Vervet monkeys shared the novel E125Q sequence variant in the *CYP3A5* gene which was regarded as SNP and two silent mutations (L295L and V296V) in exon 10, which were predicted to be polymorphisms and disease-causing, respectively (Table 2). Out of five sequence variants identified in the *GRK4* gene, two of these variants (L102R and A178V) were predicted by HSF to have the potential of interfering with splicing. Moreover, five silent variants (S61S, T180T, S580S, G648G, and T939T) and one SNP were observed in the conserved region of the *SLC4A5* gene.

Furthermore, mRNA expression of the selected RAAS genes was determined between the groups (Figure 2) and gender (Figure 3, Table S5). Statistical significance was only observed for *AGT* ($p = 0.04$, difference $= -0.4 \pm 0.1803$); however, *GRK4 and SLC4A5* had a similar trend whereby the hypertensive was highly expressed while *CYP3A5* was elevated in the normal group (Figure 2). Moreover, gender analysis only showed a significant difference in *GRK4* between Vervet males and females ($p = 0.004$, difference $= 0.9288 \pm 0.2722$), and the same trend was observed for *AGT* ($p = 0.82$, difference $= -0.08304 \pm 0.3598$) and *SLC4A5* ($p = 0.06$, difference $= 0.5764 \pm 0.2771$), although not significant (Figure 3). There

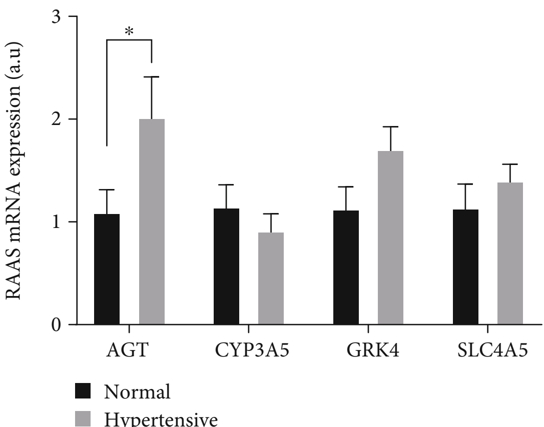

Figure 2: mRNA gene expression analysis of RAAS genes (*AGT*, *CYP3A5*, *GRK4*, and *SLC4A5*) in captive-bred Vervet monkeys. Fold change data (mean ± SD; $n = 8$) in arbitrary units (a.u) was normalized against two housekeeping genes (*PGK1* and *GAPDH*). The level of significance was set at $p < 0.05$ using GraphPad prism software. *Significant difference ($p < 0.05$).

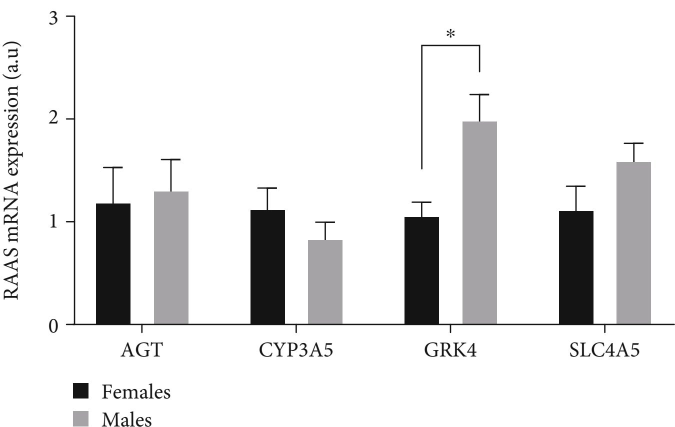

Figure 3: mRNA gene expression analysis of RAAS genes in captive-bred Vervet monkeys. The graph is showing fold-change differences (mean ± SD; $n = 8$) between females and males using GraphPad prism software. *Significant difference ($p < 0.05$) and a.u = arbitrary units.

was no significant difference in mRNA expression level between animals with R477R variants in *AGT* and T180T in *SLC4A5* against wild-type animals.

4. Discussion

About 30–50% of genetic elements contribute to hypertension [11], and these include genetic variation in RAAS genes such as *AGT*, *CYP3A5*, *GRK4*, and *SLC4A5* which are known to play a significant role in BP regulation [32]. The sequence variants identified in this study were all located in the conserved regions and anticipated to be polymorphisms, benign, and neutral by MutationTaster, PolyPhen-2, and SIFT (Table 2). Furthermore, *AGT* mRNA expression was significantly higher in the hypertensive compared to the normal group (Figure 2). Consequently, the hypertensive group had significant high level of systolic and diastolic BP compared to normal groups (Figure 1), and this may be correlated with the fact that hypertension is associated with high *AGT* expression [33, 34]. A similar trend was also observed in *GRK4 and SLC4A5*, and this can be linked with lipid accumulation in the blood vessels that are known to enhance the expression of renal angiotensin system components [35]. This may also be related to the fact that elevated *RAAS* gene expression contributes to an increase in AngII receptors in the kidney, leading to the development of hypertension [36, 37]. Moreover, *AGT* variants are associated with a higher prevalence of hypertension [23].

Evidence that links genetic variation of *CYP3A5* was identified in this study and all selected animals had E125Q variant, which was anticipated to be possibly damaging by the PolyPhen-2 (Table 2) and alteration of an exonic splicing enhancer (ESE) site by HSF. These predictions suggested that the E125Q variant might lead to the introduction of a new splice site within the exon, thereby stimulating the imbalance of *CYP3A5* metabolism activity, which enhances hypertension development. Although the L295L variant was regarded as a silent mutation in exon 10 of *CYP3A5*, it was anticipated to be disease-causing by the MutationTaster. This revealed that silent mutations can affect nucleic acid

stability, slow down translation rates, and change the structure of the protein without causing a change in amino acid [38]. A study by [39] which was conducted in the black population highlighted that *CYP3A5* variations linked to higher BP may influence salt sensitivity in hypertensive individuals. Consequently, *CYP3A5* gene expression showed higher expression in Vervet females compared to males, although this change was not significant ($p = 0.09$, $\text{difference} = -0.4171 \pm 0.2346$) (Figure 2). This was correlated to the previous studies in the human population, which showed that the *CYP3A5* gene is significantly higher in females than in males [40, 41].

To further expand on the identified mutations, two out of the five identified *GRK4* variants have been reported in human studies. These variants include L102R (Human: L65R) and A178V (Human: A142V). A study on average African Americans has reported that L65R (Vervet: L102R) and A142V (Vervet: A178V) in *GRK4* are associated with high BP levels especially in men compared to women [17]. These findings were also confirmed by a separate study conducted in South Africa [16, 21]. Additionally, another study reported that men with A142V mutation showed a statistically significant increase in diastolic BP and an increasing trend in systolic BP [21]. The *GRK4* A142V causes abnormalities in dopamine receptors due to high serine phosphorylation [20]. Similarly, the Vervet males had elevated BP levels (133.75/67.13 mmHg) and significantly higher *GRK4* expression compared to females (Figure 2). Therefore, it was speculated that *GRK4* A178V might have a similar impact in selected Vervet monkeys. The same gene expression pattern was observed in Vervet males that were highly expressing *SLC4A5*. Based on the BP, genotyping, and gene expression findings, this study further confirms the role of sequence variants and agrees with previous literature that *RAAS* polymorphisms are linked to hypertension and salt sensitivity.

5. Conclusion

Most of the identified sequence variants in this study were novel and predicted to have a significant impact such as disease-causing, possibly damaging, or deleterious effects. The genotyping results were further correlated with high BP and gene expression levels (*AGT*, *GRK4*, and *SLC4A5*) in hypertensive compared to the normal group. Based on these findings, there is a possibility that these Vervet sequence variants and gender differences affected the functioning of the *RAAS* genes. Since this is the first study that used RAAS gene variants and gene expression analysis as biomarkers of hypertension in NHPs, more research is required to further expand these findings and strengthen the use of the Vervet monkey model for cardiovascular therapy.

Data Availability

The data analysis results are included in the manuscript and the supporting documents are provided on a separate document.

Disclosure

Part of this work has been presented at the South African Association for Laboratory Animal Science (SAALAS) Conference, which was held at Northwest University Sports Village, Potchefstroom, South Africa, from 15 to 18 March 2022.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this manuscript.

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Supplementary Materials

Table S1: Clinical measurements of the captive-bred Vervet monkeys. Figure S1: the pedigree diagram of captive-bred Vervet monkeys. "*" indicates animals with normal blood pressure, and "+" indicates hypertensive animals. The circles represent females, and the square represents males. [ ] denotes wild animals that were taken from the Modderfontein farm in Potchefstroom before being housed at PUDAC. Table S2: Green monkey (Chlorocebus sabaeus) reference sequence for selected genes. Table S3: Designed PCR primers for AGT, CYP3A5, GRK4, and SLC4A5 genes, Table S4: qRT-PCR primer assays (Homo sapiens) for selected genes. Table S5: genetic variation analysis for Vervet monkeys. *(Supplementary Materials)*

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