ISSN: 0975-833X

Available online at http://www.journalcra.com

International Journal of Current Research
Vol. 11, Issue, 02, pp.1059-1064, February, 2019

DOI: https://doi.org/10.24941/ijcr.34293.02.2019

INTERNATIONAL JOURNAL OF CURRENT RESEARCH

RESEARCH ARTICLE

ADIPONECTIN GENE POLYMORPHISM SNP +276G/T AND ITS ASSOCIATION AMONG CARDIOVASCULAR SUBJECTS WITH DIABETES IN SOUTH INDIA

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ARTICLE INFO

Article History:
Received 25th November, 2018
Received in revised form
24th December, 2018
Accepted 10th January, 2019
Published online 28th February, 2019

Key Words:

Adiponectin gene,
Cardiovascular diseases,
Diabetes, SNP, South India.

ABSTRACT

Background and Objective: Adiponectin gene single nucleotide polymorphism (SNP) in the exon 3 and intron 4 is a well characterized SNP which has previously been linked to various diseases including cardiovascular diseases. The association of this gene with cardiovascular disease (CVD) is studied in various studies , hence the present study was designed to investigate the association of SNP +45T/G and +276G/T in the *AdipoQ* gene in CVD patients with and without diabetes mellitus and normal controls in North Coastal Andhra Population. **Methods:** The present study has a total 274 subjects, in which group 1 has normal controls without any complications (n=99), group 2 has cardiovascular disease subjects with diabetes (CVDDM) (n=102), and group 3 has cardiovascular disease subjects without diabetes (CVDNDM) (n=73). Patients were divided into group 2 and 3 based on the manifestation of diabetes. All the clinical, biochemical parameters were analysed for the study subjects. Adiponetin SNP were genotyped by PCR-restriction fragment length polymorphism(RFLP). **Results:** Our study has revealed a significant association of 276G/T adiponectin polymorphism with CVDDM cases in study subjects. The TT genotype against TG+GG genotype of the 276 G/T polymorphism was observed to confer increased risk to develop cardiovascular disease with diabetes (P=0.000001). TT Vs TG+GG in the given population, although the proportion of CVDDM attributable to TT genotype was 6.8% in overall study population, a similar risk factor was also observed in GG genotype against TG+TT genotype to confer the risk of developing cardiovascular disease with diabetes (P=0.0017) and GG genotype attributes nearly 50.9% in overall cases. Whereas heterozygote TG genotype does not show any significant association in CVDDM cases. **Conclusion**: The SNP 276 G/T of adiponectin gene was observed to confer increased risk to develop cardiovascular disease among diabetes subjects.

Citation: Udyama Juttada, Ramesh Mandarapu, Sudhakar Godi and Paddaiah Gangisetti. **2019.** "Adiponectin gene polymorphism SNP +276g/t and its association among cardiovascular subjects with diabetes in south India", *International Journal of Current Research*, 11, (02), 1059-1064.

INTRODUCTION

Cardiovascular disease (CVD) is the leading cause of mortality and morbidity in patients with diabetes. Compared with nondiabetic individuals, diabetic patients have a 3-fold higher risk of developing atherosclerosis and its clinical complications (Kannel, 1987). Both coronary artery disease and type 2 diabetes mellitus have a strong genetic basis, they often occur together, and epidemiologic evidence suggests that this occurrence also has a genetic basis (Mitchell, 2002). With the growing worldwide obesity epidemic, type 2 diabetes mellitus and hypertension leading to premature cardiovascular events, are increasingly prevalent. Diabetes among them is a significant public health concern and more aggressive management of the condition and its complications, particularly cardiovascular disease, is required.

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Endothelial cell dysfunction is now known to be present at the earliest stages of metabolic syndrome, and insulin resistance and may precede the clinical diagnosis of type 2 diabetes mellitus by several years. The current focus on endothelial cell function as a potential target of pharmacotherapy in the management of cardiovascular disease in diabetics seems warranted (Highlander, 2010). Recent advances in the field of molecular biology have led to a better understanding of the pathological mechanisms of cardiovascular disease (Cambien, 1999). The impact of these findings will shape the future of treatment modalities for cardiovascular disorders. Postulated targets and biological rationale of new techniques are being developed in a race towards molecular therapies for vascular diseases. Whether it is modulation of transmembrane cell receptors or phenotypic changes via vectors that mediate gene transfer, there is no doubt that molecular strategies will be an integral part of the future therapies. According to some authors, the classic cardiovascular risk factors such as smoking, hypertension, dyslipidemia, diabetes, obesity, sedentary lifestyle, and dietary factors do not completely

explain the differences in the prevalence of cardiovascular disease between different populations (Masia, 1998 and Pyorala, 2000). Additional markers such as adipocytokine and fasting glucose levels may be of great help in risk stratification and improving treatment directed toward specific populations. Therefore, adiponectin may be considered emerging risk factors. Adiponectin is an adipose tissue derived adipokine, first described in 1995. Over the past two decades, numerous studies have elucidated the physiological functions of adiponectin in obesity, diabetes, inflammation, atherosclerosis, and cardiovascular disease (Ryo, 2004 and Knobler, 2006). Adiponectin, elicited through cognate receptors, suppresses glucose production in the liver and enhances fatty acid oxidation in skeletal muscle, which together contributes to a beneficial metabolic action in whole body energy homeostasis. Beyond its role in metabolism, adiponectin also protects cells from apoptosis and reduces inflammation in various cell types via receptor-dependent mechanisms (Whitehead, 2006). Adiponectin, as a fat-derived hormone, which fulfills a critical role as an important messenger to communicate between adipose tissue and other organs. A better understanding of adiponectin actions, including the pros and cons, will advance our insights into basic mechanisms of metabolism and inflammation, and potentially pave the way towards novel means of pharmacological intervention to address path physiological changes associated with diabetes, atherosclerosis, and cardiometabolic disease (Han, 2009). Because of adiponectin major role in vascular systems our study focus on south Indians population who are known to have increased susceptibility to diabetes compared to Europeans (McKeigue, 1989). It has been shown that for any given body mass index (BMI), Asian Indians have higher body fat (particularly visceral adiposity), higher plasma insulin levels and greater insulin resistance compared to Europeans (Mohan , 1986 and Sharp , 1987). The diabetes is proved to be a major risk factor for CVD in many studies from South Indian population. In this study, we applied the recently evolved Indian Diabetes Risk Score (IDRS) in subjects with different grades of glucose intolerance and evaluated its usefulness as an indicator of cardiovascular risk in Indians).because of this high significance our study has been distributed the cases on their diabetic profiles and wanted to observe the pattern of significance of the different biochemical and molecular markers in cardiovascular patients with and without diabetes mellitus Asian also reported to have lower adiponectin levels (Abate, 2004), which might leads to some of the metabolic abnormalities which can be explained by genetic factors.

MATERIALS AND METHODS

In the present cross-sectional study, we enrolled a total of 274 subjects in each group , Group 1 Healthy controls consisted of non-diabetic subjects (n = 99; M/F:122/96). Group 2 cardiovascular patients with T2DM (CVDDM)(n =102; M/F : 111/85). Group 3 cardiovascular patients without T2DM (CVDNDM)(n =73; M/F : 203/139). The study protocol was approved by the institutional ethics committee of King George Hospital Andhra Pradesh during the period from April 2009 to June 2011 and written informed consent was obtained from all enrolled study subjects in accordance with the principles of the Declaration of Helsinki. The healthy controls were mostly blood donors and hospital staffs. Anthropometric measurements including age, height and weight were obtained using standardized techniques. The body mass index (BMI) was calculated as the weight in kilo-grams divided by the square of height in meters. FPG was measured by glucose oxidase peroxidase method, serum cholesterol by cholesterol oxidase-peroxidase amidopyrine method, serum triglycerides using glycerol phosphate oxidase-peroxidase amidopyrine method, high density lipoprotein cholesterol (HDL-c) by the direct method (polyethylene glycol pretreated enzymes) and creatinine with Jaffe's method was measured using a Hitachi-912 Autoanalyser (Hitachi, Mannheim, Germany). The intra and inter assay coefficients of variation for the biochemical assays ranged between 3.1% and 5.8% respectively. Low-density lipopro-tein cholesterol (LDL-c) was calculated using the Friedewald equation (Pischon, 2004). All the biochemical estimations were done in an NABL accredited laboratory.

Genotyping of AdipoQ 45 T/G and 276 G/T promoter polymorphism: 5 mL of venous blood was collected from the patients and healthy subjects in k3 EDTA coated tubes (Greiner Bio-One, North America Inc., North Carolina, USA). Genomic DNA was extracted from peripheral blood using the standard phenol-chloroform extraction method. After extraction, concentration and purity of DNA were estimated spectrophotometrically, quality of DNA was also determined on 0.8% agarose gel electrophoresis and DNA was stored at −20 ◦C until further analyses. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was used to genotype the promoter polymorphisms of adipoQ gene. Primers used for genotyping 45 T/G and 276 G/T were mentioned in the Table 1 (Sigma-Aldrich Chemical Co, St. Louis, Missouri, USA). For 45 T/G and 276 G/T the reaction mixture of the total volume of 20 micro liter (µl) included 5 µl (100 ng) of genomic DNA, 10µl of nuclease-free water, 2 µl of (10×) PCR buffer, 2 µl of (2 mM) dNTPs, 0.3 µl of (10µM) forward and reverse primers and 0.3 µl of (0.2 U/ µL) Aura Taq DNA Polymerase 2X Mastermix Red (Aura Biotechnologies Pvt Ltd, Mastermix, Ayanambakkam, Chennai, India). Amplification was performed using a S1000 thermal cycler (BioRad Laboratory, Hercules, California, USA) according to the protocol. For 45 T/G polymorphism initial incubation of 95°C for 5 min, followed by 35cycles of denaturation at 95°C for 30 sec, annealing at 60°C for 30 sec, and extension at 72 °C for 30 sec, followed by a final incubation at 72°C for 10 min. Further, the amplicons were digested using Ava I restriction enzyme (New England Biolabs, Beverly, MA). For 276 G/T polymorphism initial incubation of 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec, and extension at 72°C for 30 sec, followed by a final incubation at 72°C for 10 min. Further, the amplicons were digested using Hinf I restriction enzyme (New England Biolabs, Beverly, MA) 5µl of the amplified products in both PCR reactions were digested with 5U of the corresponding restriction enzyme in a total reaction volume of 25µl as per the manufacturer's instruction. The digested products were resolved on a 2.5% agarose gel stained with ethidium bromide and visualized under UV transilluminator. The size was determined by comparison to a molecular weight standard 1 kb Plus (Life Technologies Corpora-tion, Carlsbad, CA). To ensure that the genotyping was of adequate quality, we performed duplicate genotyping in 20% of the randomly selected samples.

Statistical analysis: Statistical calculations were performed using SPSS (version 20.0; SPSS, Chicago, IL, USA). Normally distributed data are presented as mean ± S.D. The Hardy-Weinberg equilibrium was tested with the $\chi 2$ test.

Table 1. Details of Primer sequences, amplicon sizes and the restriction enzymes used in the study

Region	Reference SNP number	Primers (5'-3')	Anneling temperature	Product size (bp)	Expected band patterns (bps)	Restriction enzymes
SNP: 45T/G	rs2241766	Forward: 5'-TGT GTG TGT GGG GTC TGT CT-3' Reverse: 5'-TGT GAT GAA AGA GGC CAG AA-3'	60	305	305, 200 and 105	Ava I
SNP: 276G/T	rs1501299	Forward: 5'-CTA CAC TGA TAT AAA CTA TAT GGA G-3' Reverse: 5'-CCC CAA ATC ACT TCA GGT TG-3'	56	107	107, 82 and 25	Hinf I

Table 2. Clinical and biochemical characteristics of the study subjects

Clinical parameters	Group 1 Controls (n= 99)	Group 2 CVD-DM (n=102)①	Group 3 CVD-NONDM (n=73)②
Gender (M/F)	73:26	66:36	52:21
Age (years)	46.1±14.9	58.11±10.3***	51.8±14.0**
BMI (Kg/m2)	24.2±3.3	27.17±4.3**	25.8±3.9***
SBP (mm Hg)	109.2±17.9	129.8±25.4***	128.9±10.8***
DBP (mm Hg)	79.58 ± 6.55	90.2±7.5*	95.4±8.1**
Fasting plasma glucose (mg/dL)	95.6 ± 7.0	156.0 + 37.01***	99.6 ± 7.0
Postprandial plasma glucose(mg/dL)	120.8 ± 13.4	211.9 + 46.71***	120.8 ± 13.4
Glycated Hemoglobin (%)	13.0±2.1	12.0±2.2	12.5±2.5
Total serum Cholesterol(mg/dL)	171.4±16.4	203.4±92.5***	203.4±92.5**
VLDL (mg/dL)	38.7±19.1	45.7±19.2***	26.83±13.5
HDL-cholesterol (mg/dL)	42.9±4.2	29.0±2.9***	30.1±10.9***
LDL-cholesterol (mg/dL)	121.3±51.4	164.5±42.1	117.6±51.43*
Urea (mg/dL)	20.3±6.1	22.8 ± 5.6	31.6 + 15.4*
Creatinine (mg/dL)	0.8±0.1	0.9±0.1	1.1 + 0.2
Smoking (%)	27%	63%	46%
Family history of CVD (%)	NIL	39%	24%

All data are reported as mean± SD for continuous variables. BMI-Body mass index; SBP-Systolic Blood Pressure; DBP-Diastolic Blood Pressure;HDL- High Density Lipoprotein; LDL-Low Density Lipoprotein .
* P < 0.05, **P < 0.01, ***P < 0.001.
① : indicates comparison was made between controls and CVD-DM subjects.
② : indicates comparison was made between controls and CVD-NON DM.

Table 3. Distribution of genotype and allele frequencies of AdipoQ SNP 45 T/G and 276G/T in the study subjects

Genotype (SNP)	Controls (n=99)	CVD-DM (n=102)	CVD-NONDM (n=73)
45T/G			
TT	75(75.9%)	76(74%)	57(78%)
GG	20(20.2%)	5(4.9%)	3(4.1%)
TG	4(4.0%)	21(20%)	13(17.8%)
276G/T			
TT	38(38.3%)	7(6.8%)	25(34.2%)
TG	32(32.2%)	43(42.1%)	28(38.5%)
GG	29(29.2%)	52(50.9%)	20(27.3%)

Values are numbers (percentage).

Genotype distribution and allele frequencies were compared among groups using a $\chi 2$ test of independence with 2 × 2 contingency and z statistics. Kruskal–Wallis test was used for multiple parameters that did not exhibit the normal distribution. Where appropriate, the odds ratio (OR) with 95% confidence interval (CI) were calculated. P value less than 0.05 were considered significant.

RESULTS

Table 2 shows the selected anthropometric and biochemical characteristics of the study subjects. Compared to CVD DM subjects, parameters such as BMI, Systolic Blood Pressure (SBP), FPG, PPG, total cholesterol, HDL and VLDL were found to be significantly higher while the LDL, hemoglobin, urea and creatinine were not significant. Similarly when compared to CVD NON DM subjects parameters such as age, BMI , Systolic Blood Pressure (SBP), LDL, HDL and total cholesterol were found to be significantly higher while the VLDL and hemoglobin does't show much significance. The percentage of smokers in patients (63%) was found to be high when compared to controls (46%).

The percentage of individuals with family history of Cardiac diseases in patients was 39%. Controls were selected without any such histories as they are the risk factors for CVD.

Genetic association of Adipo Q SNP's in 45 T/G and 276 G/T with disease phenotype

Adiponectin markers 45T/G & 276 G/T marker was analyzed using the PCR RFLP procedure, and there genotypic data was shown in table 4 which noted that only 276G/T markers showed significant association with CVD-DM cases, which conclude that the polymorphism in the 276 G/T marker in CVD-DM cases might be due to the predisposition of diabetes in CVD patients. Our study has revealed a significant association of 276G/T adiponectin polymorphism with CVD-DM cases in north coastal andhra population. The TT genotype against TG+GG genotype of the 276 G/T polymorphism was observed to confer increased risk to develop cardiovascular disease with diabetes millitus. (P=0.000001). Although the proportion of CVD-DM attributable to TT genotype was 6.8% and GG genotype attributable to nearly 50.9% in overall cases of the study population, TT genotype Vs TG+GG genotype in the given

Table 4. Test of Association, Relative Risk, Odds Ratio and 95% Confidence Interval Estimates of SNP Phenotypes

Genotype (SNP)	CVD DM vs Controls		CVD NONDM vs Controls	
45 T/G	OR(95% CI)	p-value	OR(95% CI)	p-value
TT vs TG+GG	0.94 (0.47 - 1.86)	0.8379	1.14(0.52 - 2.49)	0.7213
TG vs TT+GG	1.02 (0.49 - 2.15)	0.9458	0.86 (0.37 - 1.98)	0.6935
GG vs TG+TT	1.22 (0.27 - 5.63)	0.7678	1.02 (0.17 - 5.61)	0.9819
276 G/T				
TT vs TG+GG	0.12 (0.04- 0.30)	0.000001***	0.84 (0.42 - 1.65)	0.577
TG vs TT+GG	1.53 (0.82 - 2.83)	0.1495	1.30 (1.66 - 2.57)	0.4119
GG vs TG+TT	2.51 (1.35 - 4.69)	0.0017***	0.91 (0.44 - 1.88)	0.7854

Figures in bold were significant (P < 0.05). Controls, subjects with normal glucose tolerance.
Odds ratio (OR), 95% class interval (CI)
* P < 0.05, ***P < 0.001

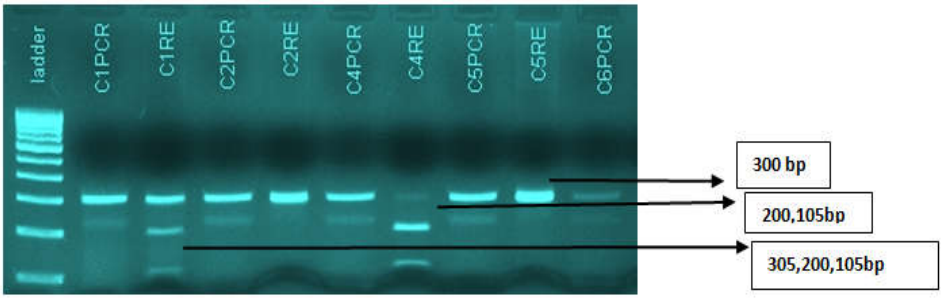

Figure 1. 45 T/G polymorphism of the Adipo Q gene

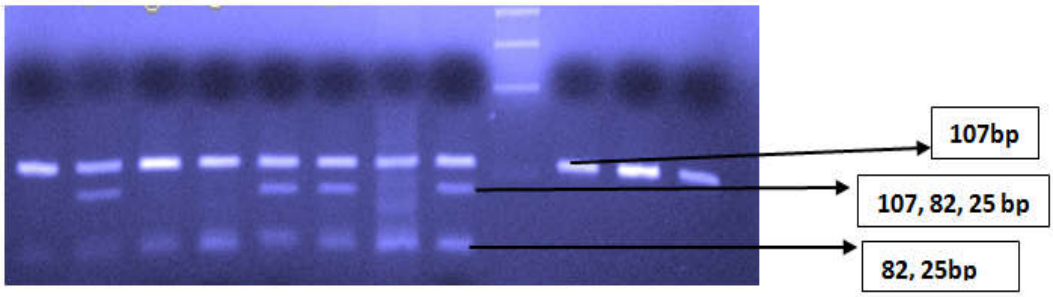

Figure 2. 276 G/T polymorphism of the Adipo Q gene

genotype against TG+TT genotype to confer the risk of population has a similar risk factor as was observed with GG genotype against TG+TT genotype, to confer the risk of Developing cardiovascular disease with diabetes mellitus (P=0.0017) developing cardiovascular disease with diabetes mellitus (P=0.0017) and GG genotype attributes nearly 50.9% in overall cases. Where as heterozygote TG genotype does not show any significant association with CVD-DM cases. Interestly when the 276G/T polymorphism has analysed within CVD-NDM and normal controls, there was no association observed with all the 3 genotypes (TT=P=0.57) TG (P=0.411), GG (P=0.78) the T/T genotype construct (34.2% of overall population &GG (27.3%).

DISCUSSION

Several studies have represented individuals with diabetes as an independent risk factor for CVD in Indian and American population (De Simone , 2010 and Ranjith, 2008), also opined that, diabetes is a risk factor for CVD in South Indian population, in these studies (Guptha, 2008), they applied the recently evolved Indian Diabetes Risk Score (IDRS) in subjects with different grades of glucose intolerance and evaluated its usefulness as an indicator of cardiovascular risk in Indians, because of this high significance our study has been distributed the cases on their diabetes profiles and wanted to observe the pattern of significance of the different biochemical and molecular markers in cardiovascular patients with and without diabetes mellitus. The present study showed neither the distribution of alleles nor the genotypes of 45T/G adiponectin polymorphism significant among cases and controls. The results of the present investigation are in concordance with few studies in the past that had also demonstrated no association with 45T/G among CVD subjects (Vasseur, 2003) In a study among french caucasians have found no significance when studied among normoglycemic and type 2 diabetes subjects (Bacci, 2004). Similar results were also demonstrated between cases and controls for non-fatal MI or fatal CHD in men and women in a study by pischon (Pischon, 2004). Among the SNPs in the Adiponectin gene, according to a japanese study SNP located at 276 bp downstream of the translational start site was directly associated with decreased plasma adiponectin level, greater insulin resistance, and an increased risk of type 2 diabetes (Vasseur, 2002) i.e the subjects, with both 2 alleles of SNP 276 are the G (G/G genotype), had an approximately doubled risk for developing type 2 diabetes as compared with those with the T/T genotype, which makes subjects prone to genetically decreased adiponectin levels and thus susceptible to type 2 diabetes. Same results was observed in a study by Alireza esteghamati (Esteghamati, 2003), where a decreased risk of CAD in SNP +276 G>T after adjustment for potential confounding factors.

The protective effect conferred by the SNP +276 G>T appears to be allele dose dependent with a multiplicative effect on the odds ratio scale. The present study showed a very high significant association of 276 G/T SNP among CVD-DM subjects. Interesting when the 276 G/T polymorphism has been analyzed within CVD-NON DM cases and normal controls, there was no association observed with all the 3 genotypes which confers that the risk is more because of the presence of the diabetes. Evidence indicates that the putative functional SNP +276G/T and promoter polymorphisms in the Adipo Q gene are associated with circulating adiponectin levels. Thus these polymorphisms functionally regulate adiponectin promoter activity and its protein levels in blood. Knowledge of cellular mechanism how these polymorphisms regulate plasma adiponectin levels is still limited. +276(G/T), as a putative functional SNP, is found to be associated with plasma adiponectin levels in Type 2 Diabetes, (Hara, 2002) and Type 1 diabetes and also in nonalcoholic steatohepatitis (NASH) (Musso, 2008), the single nucleotide polymorphism G276T appears to be associated with lower levels of adiponectin and with the earlier onset of coronary artery disease (Filippi, 2005). Other two studies (Qi , 2005 and Pischon, 2004) which reported an association of this variation with decreased risk of CAD under a recessive mode of inheritance. Whereas Filippi et al (Qi, 2005) reported an association of the variant with increased CAD risk in individuals without diabetes. Interestingly, Lacquemant et al., 2004 (Lacquemant, 2004), reported no association between the +276G>T variant and CAD risk in diabetic patients, and also a study by Hegener et al (28) also showed no evidence for an association of either the +276G>T variation with risk of incident MI and ischemic stroke.

Conclusions

In the present study we found a strong association between the +276G>T SNP and CVD-DM subjects, Furthermore, carriers of the T allele had significantly lower serum adiponectin levels. In view of these results, it could be speculated that the adiponectin gene variant, or a mutation in linkage with it, determines lower adiponectin gene expression, causing in turn an increased risk to develop insulin resistance, atherosclerosis and cardiovascular disease. Our data provide the first evidence for an association between T allele of 276G>T SNP and decreased risk of coronary artery disease with diabetes mellitus (CVD-DM) in north coastal Andhra population.

Acknowledgements

This work is done at Department of Human Genetics Andhra University we thank all the technical staff for their help in carring out the experiment and author would like to thank all our King George hospital visakhapatnam staffs for cooperating in collecting patients data and samples. We thank Trims biosciences institute for their typographical assistance and also study patients for their cooperation.

Conflict of Interest statement: The author declares that there is no duality of interest associated with this manuscript.

Funding statement: No external funding is provided to these study.

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