

# Association of Genetic Variability in Selected Genes in Patients With Deep Vein Thrombosis and Platelet Hyperaggregability

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## Abstract

The aim of this study was to evaluate the genetic variability of the selected single nucleotide polymorphisms (SNPs) and examine the association between these SNPs and risk for deep vein thrombosis (DVT) in patients with sticky platelet syndrome (SPS). We examined 84 patients with SPS and history of DVT and 101 healthy individuals. We were interested in 2 SNPs within platelet endothelial aggregation receptor 1 (PEAR1) gene (rs12041331 and rs12566888), 2 SNPs within murine retrovirus integration site 1 gene (rs7940646 and rs1874445), 1 SNP within Janus kinase 2 gene (rs2230722), 1 SNP within FCER1G gene (rs3557), 1 SNP within pro-platelet basic protein (rs442155), 4 SNPs within alpha2A adrenergic receptor 2A (ADRA2A; rs1800545, rs4311994, rs1195419, and rs553668), and 1 SNP within sonic hedgehog gene (rs2363910). We identified 2 protective SNPs within PEAR1 gene and 1 risk SNP within ADRA2A gene (PEAR1: rs12041331 and rs12566888; ADRA2A: rs1800545). A haplotype analysis of 4 SNPs within ADRA2A gene identified a risk haplotype aagc ( $P = .003$ ). Moreover, we identified 1 protective haplotype within PEAR1 gene (AT,  $P = .004$ ). Our results support the idea that genetic variability of PEAR1 and ADRA2A genes is associated with platelet hyperaggregability manifested as venous thromboembolism. The study also suggests a possible polygenic type of SPS heredity.

## Keywords

hyperaggregability, single nucleotide polymorphism, platelet, deep vein thrombosis

## Introduction

Platelet hyperaggregability after low concentrations of platelet agonists adenosine diphosphate (ADP) and/or epinephrine (EPI), referred to as sticky platelet syndrome (SPS), was first described by Holiday at the Ninth Conference on Stroke and Cerebral Circulation in Arizona in 1983.<sup>1</sup> Clinical symptoms of SPS include unexplained arterial and venous thrombotic events, commonly occurring in stressful situations and frequently recurrent under oral anticoagulant therapy.<sup>1-4</sup> Furthermore, there is a causal relation between SPS and abortion.<sup>1,5,6</sup>

Sticky platelet syndrome is diagnosed by the aggregometry (platelet aggregation measurement that is performed by aggregometer) after the platelet activation by inducers—ADP and/or EPI. By the results of aggregometry, SPS is classified as type I (hyperaggregation after both ADP and EPI), type II (hyperaggregation after EPI alone), and type III (hyperaggregation after ADP alone). Sticky platelet syndrome type II seems to be the most common one.<sup>1,2</sup>

Since both genders are affected, SPS has a clear autosomal pattern of inheritance, although the exact genetic cause has not been identified yet. It has been suggested that the defects of the

platelet membrane glycoproteins or intracellular signal pathways involved in platelet activation and aggregation are responsible for the disorder.<sup>7</sup>

Several candidate gene studies have examined the association of genetic variants in specific genes with platelet aggregation.<sup>8</sup> A genome-wide association study in European Americans identified 7 loci associated with agonist-induced platelet aggregation.<sup>9</sup> Based on these results, we used a number of single nucleotide polymorphisms (SNPs) from 7 genes (platelet endothelial aggregation receptor 1 [PEAR1], murine

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retrovirus integration site 1 [MRVI1], janus kinase 2 [JAK2], FCER1G, pro platelet basic protein [PPBP], alpha2A adrenergic receptor [ADRA2A], and sonic hedgehog [SHH]).

Platelet endothelial aggregation receptor 1 is a type 1 membrane protein, which is expressed on platelets and endothelial cells. It has been shown that genetic variants in PEAR1 associate with increasing overall platelet aggregation and reduced responsiveness to aspirin in patients with premature cardiovascular disease.<sup>10</sup> Murine retrovirus integration site 1 homolog, which showed both ADP- and EPI-induced associations, has prior evidence of functions in platelet aggregation. In mice, MRVI1 plays a direct role in the inhibition of platelet aggregation and in vivo thrombosis.<sup>11</sup> Janus kinase 2 is an intracellular nonreceptor tyrosine kinase that transduce cytokine-mediated signals. The JAK2-STAT3 pathway is involved in collagen-induced platelet activation.<sup>12</sup> High affinity immunoglobulin epsilon receptor subunit gamma (FCER1G) is involved in collagen-mediated platelet activation.<sup>13</sup> Pro-platelet basic protein is a platelet-derived growth factor that belongs to the CXC chemokine family. This growth factor is a potent chemoattractant and activator of neutrophils. It is a protein that is released in large amounts from platelets following their activation.<sup>14</sup> Alpha2A adrenergic receptor on platelets interacts with EPI, which has a key role in regulating platelet functions. There is familial clustering of interindividual variations in the EPI-induced platelet aggregation, the molecular basis of which, however, has not been fully understood.<sup>15</sup> Sonic hedgehog is a protein that in humans is encoded by the SHH. It has been shown that rs2363910 polymorphism within this gene is associated with ADP-induced aggregation.<sup>9</sup>

The aim of this study was to evaluate the variability of selected genes in group of patients with SPS manifested as deep vein thrombosis (DVT) compared to control individuals and to determine the relationship between selected regions of the genes and SPS.

## Material and Methods

### Study Population and Inclusion/Exclusion Criteria

The local Ethical Committee of the Jessenius Faculty of Medicine in Martin approved the study (Number EK950/2011). All study participants agreed to participate in the project and signed a written informed consent in accordance with the Declaration of Helsinki, including healthy blood donors.

All patients were enrolled into this study between March 2011 and September 2017. Patients were initially examined and tested at the Department of Haematology and Transfusion Medicine in Martin University Hospital. Inclusion criteria were platelet hyperaggregability induced by at least 2 concentrations of EPI and/or ADP during the light transmission platelet aggregometry (LTA) according to the method by Mammen,<sup>3</sup> documented at least twice by 2 distinct assessments with an interval of at least 1 month; DVT verified by Doppler ultrasonography; lack of triggering risk factors for the DVT, including surgery, cancer, immobilization, pregnancy, puerperium, hormonal

therapy, oral contraceptives use, and travel longer than 4 hours; lack of strong known genetic risk factors for DVT, including antithrombin, protein C or S deficiencies, factorII 20210A or homozygosity for factor V Leiden which were tested at our department during this study; lack of diabetes mellitus and autoimmune disease; and European origin and signing of the informed consent. Patients who met at least one of the exclusion criteria were ineligible for the study, hence they were not included: platelet hyperaggregability induced by only 1 concentration of EPI and/or ADP agonists during LTA according to the method by Mammen<sup>3</sup> or documented only once; unclear or insufficiently documented diagnosis of DVT or pulmonary embolism; secondary venous thromboembolism (VTE) defined as DVT and/or pulmonary embolism occurring within 3 months after exposure to exogenous risk factors for VTE (as mentioned above); documented strong known genetic risk factors for VTE (as mentioned above); acute episode of VTE or less than 8 weeks after acute episode of VTE; pregnancy or puerperium; documented active cancer or history of malignancy for less than 5 years, liver or kidney failure, chronic inflammation or autoimmune disease; and lack of informed consent.

Furthermore, 101 randomly chosen age-matched healthy blood donors from the same region of Zilina, the northwestern part of Slovakia, were involved as control individuals. All control individuals were Caucasians of European origin, with negative personal and family history of the VTE and normal platelet aggregability after stimulation by an EPI and/or ADP agonist according to the method by Mammen.<sup>3</sup>

All blood samples in both study groups were taken outside the menstrual period or other active bleeding. Inflammatory process was excluded by measuring C-reactive protein level (normal value < 5.0 mg/L) and normal white blood cell count (normal range  $4-10 \times 10^9/L$ ).

The demographic and clinical characteristics of the patients and controls, including medication, family and personal history of VTE, and acquired risk factors for VTE were recorded during face-to-face interview conducted by a medical doctor.

### Diagnostics of SPS

The antecubital venous blood was collected into the tubes of 3.2% buffered sodium citrate (anticoagulant-blood ratio 1:9) to assess the platelet aggregation. The samples were processed and analyzed within 2 hours after sampling. Platelet aggregability was tested with platelet-rich plasma using platelet aggregometry (PACKS-4 aggregometer, Helena Laboratories, USA) for Helena Laboratories.) according to Mammen. Each sample was tested with 3 low concentrations of ADP (2.34, 1.17, and 0.58  $\mu\text{mol/L}$ ) and EPI (11.0, 1.1, and 0.55  $\mu\text{mol/L}$ ). The aggregation was assessed photometrically by the platelet aggregometer with the use of platelet-rich plasma.

**Table 1.** Characteristics of the Selected SNPs.

Ref SNP ID Number	Gene	Chromosome	Position Within Gene	SNP Sequence	Major/Minor Allele
rs12041331	PEAR1	1	Intronic	CTTCC[G/A]TCACC	G/A
rs12566888	PEAR1	1	Intronic	TCCAG[G/T]ATAGG	G/T
rs7940646	MRVII	11	Intronic	GACAG[G/A]CCCAC	G/A
rs1874445	MRVII	11	Intronic	GTTTT[G/A]ACTCA	G/A
rs2230722	JAK2	9	Exonic	GTGCA[C/T]GGATG	C/T
rs3557	FCER1G	1	3' Regulatory region	CCCTG[T/G]TAAAG	T/G
rs442155	PPBP	4	Intronic	CAACT[A/G]AATCA	A/G
rs1800545	ADRA2A	10	5' Regulatory region	AAGGC[G/A]CCCAC	G/A
rs4311994	ADRA2A	10	Intronic	CTGGC[C/T]GGCCA	C/T
rs11195419	ADRA2A	10	3' Regulatory region	TTTGG[C/A]CACTC	C/A
rs553668	ADRA2A	10	3' Regulatory region	AAGAA[G/A]TTTTT	G/A
rs2363910	SHH	7	Intronic	CTGTT[G/T]ACCAT	G/T

Abbreviations: ADRA2A, alpha2A adrenergic receptor 2A; C, cytosine; G, guanine; JAK2, Janus kinase 2; MRVII, murine retrovirus integration site 1; PEAR1, platelet endothelial aggregation receptor 1; PPBP, pro-platelet basic protein; SHH, sonic hedgehog; A, adenine; SNP, single nucleotide polymorphism; T, thymine.

### DNA Analysis

Antecubital venous blood used for DNA analysis was collected into tubes containing 5.4 mg K2EDTA (spray-coated). Samples were processed within 2 hours of collection and stored, if necessary, at  $-20^{\circ}\text{C}$ . DNA was extracted from peripheral blood leukocytes. Isolation of genomic DNA from whole blood was performed with SiMax<sup>TM</sup> Genomic DNA Extraction kit (SBS Genetech Co, Ltd, China) according to manufacturer's instructions. High-resolution melting analysis on LightCycler 480 II (Roche Diagnostics, Mannheim, Germany) was used for SNP genotyping. The selection and designing of primer sequences were performed by Primer3 software.<sup>16</sup>

### Gene Analysis

We selected representative SNPs within suspected genes for platelet hyperaggregability: 2 SNPs within PEAR1 gene (rs12041331 and rs12566888), 2 SNPs within MRVII gene (rs7940646 and rs1874445), 1 SNP within JAK2 gene (rs2230722), 1 SNP within FCER1G gene (rs3557), 1 SNP within PPBP (rs442155), 4 SNPs within ADRA2A (rs1800545, rs4311994, rs11195419, and rs553668), and 1 SNP within SHH gene (rs2363910). For the selection of ADRA2A tag SNPs, data from the 1000 Genomes Project<sup>17</sup> and an adopted algorithm implemented in Haploview 4.2 were used.<sup>18</sup> The basic characteristics of analyzed SNP including nucleotide sequence is given in Table 1.

### Statistics

We used IBM SPSS for Windows 16.0 (Statistical Package for the Social Sciences, Version 16.0, SPSS Inc for statistical analysis, USA). The haplotype association analysis was performed using SNP and variation Suite 7 (SNP & Variation Suite v7.8, Golden Helix, Bozeman, Montana). The Fisher exact test was used to estimate a significance of deviation from Hardy-Weinberg equilibrium (HWE) and to execute basic allelic association. The *P* values less than .05 were considered statistically

significant. Odds ratios (ORs) with 95% confidence intervals (95% CIs) were used to assess risk. A haplotype frequency was estimated using the expectation-maximization algorithm.

### Results

We examined 84 patients with SPS type II, 24 (28.6%) men and 60 (71.4%) women with age of  $43.2 \pm 12.3$  years and 101 healthy individuals, men 47 (46.5%) and women 54 (53.5%) with the age of  $38.5 \pm 12.99$  years.

In sum, we examined 12 SNPs. One was localized in coding region of JAK2 gene. Seven selected polymorphisms were recognized in intronic regions (rs12041331, rs12566888, rs7940646, rs1874445, rs442155, rs4311994, and rs2363910). In addition, we had examined SNPs in regulatory regions on 5' and 3' ends of selected genes (3' end: rs3557, rs11195419, and rs553668; 5' end: rs1800545).

All mentioned variations were tested for the deviation from HWE. The minor allele frequencies for both 2 SNPs within PEAR1 gene were significantly lower in patients having SPS with history of DVT compared to healthy individuals (rs12041331: 0.04 vs 0.11, OR: 0.356, 95% CI: 0.148-0.855; rs12566888: 0.05 vs 0.11, OR: 0.389, 95% CI: 0.169-0.894). We sought to assess the frequency of subgroup analysis by sex/gender in these 2 SNPs, and we did not find any significant difference compared to main analysis (rs12041331: male, 0.04 vs 0.12, OR: 0.328, 95% CI: 0.07-0.893, female, 0.04 vs 0.10, OR: 0.383, 95% CI: 0.129-0.932; rs12566888: male, 0.04 vs 0.13, OR: 0.328, 95% CI: 0.070-0.891, female, 0.05 vs 0.11, OR: 0.421, 95% CI 0.152-0.548). In addition, we found that 1 polymorphism in 5' regulatory region of ADRA2A gene was significantly more frequent in patients having SPS with history of DVT compared to healthy controls (rs1800545: 0.12 vs 0.06, OR: 2.077, 95% CI: 1.006-4.286). The gender subgroup analysis did not show any difference compared to main analysis (male, 0.12 vs 0.06, OR: 1.995, 95% CI: 1.009-4.106, female, 0.14 vs 0.05, OR: 3.400, 95% CI: 1.209-9.560), see Table 2.

**Table 2.** Frequency of Selected Alleles in Patients With SPS and Control Individuals.

RefSNP		Freq. (SPS)	Freq. (Control)	Fishers HWE P (SPS)	Fishers HWE P (Control)	Fishers Exact P Allelic Association	OR Minor Allele	95% CI
rs12041331	All	0.04	0.11	1.000	0.600	0.017	0.356	0.148-0.855
	Male	0.04	0.12	1.000	1.000	0.020	0.328	0.070-0.893
	Female	0.04	0.10	1.000	1.000	0.045	0.383	0.129-0.932
rs12566888	All	0.05	0.11	1.000	0.355	0.022	0.389	0.169-0.894
	Male	0.04	0.13	1.000	1.000	0.022	0.328	0.070-0.891
	Female	0.05	0.11	1.000	1.000	0.049	0.421	0.152-0.548
rs7940646	All	0.29	0.30	0.792	1.000	0.733	0.925	0.590 -1.450
	Male	0.29	0.35	0.127	0.346	0.477	0.761	0.359-1.616
	Female	0.28	0.26	0.524	0.306	0.683	1.130	0.629-2.029
rs1874445	All	0.36	0.38	1.000	0.836	0.720	0.925	0.606-1.414
	Male	0.29	0.43	0.621	0.550	0.120	0.556	0.264-1.171
	Female	0.39	0.34	0.786	0.763	0.443	1.235	0.720-2.121
rs2230722	All	0.28	0.33	0.184	0.260	0.281	0.783	0.501-1.223
	Male	0.21	0.35	0.031	0.024	0.080	0.486	0.215-1.099
	Female	0.31	0.32	1.000	0.762	0.872	0.955	0.547-1.669
rs3557	All	0.06	0.10	1.000	0.239	0.166	0.576	0.262-1.267
	Male	0.04	0.10	1.000	1.000	0.335	0.411	0.085-1.981
	Female	0.07	0.10	1.000	0.077	0.337	0.630	0.243-1.629
rs442155	All	0.11	0.09	1.000	0.564	0.560	1.227	0.617-2.441
	Male	0.12	0.10	1.000	0.344	0.591	1.349	0.450-4.042
	Female	0.10	0.08	0.456	1.000	0.664	1.222	0.494-3.025
rs1800545	All	0.12	0.06	0.348	1.000	0.044	2.077	1.006-4.286
	Male	0.12	0.06	0.349	1.000	0.044	1.995	1.009-4.106
	Female	0.14	0.05	0.586	1.000	0.015	3.400	1.209-9.560
rs4311994	All	0.11	0.08	0.588	1.000	0.354	1.395	0.688-2.829
	Male	0.08	0.10	1.000	1.000	1.000	0.859	0.250-2.946
	Female	0.10	0.06	1.000	1.000	0.299	1.665	0.630-4.398
rs11195419	All	0.07	0.11	0.344	0.599	0.214	0.629	0.302 -1.313
	Male	0.10	0.12	1.000	1.000	0.819	0.877	0.286-2.688
	Female	0.06	0.10	0.169	1.000	0.224	0.546	0.204 -1.464
rs553668	All	0.10	0.12	0.150	1.000	0.384	0.745	0.384 -1.448
	Male	0.12	0.12	0.021	1.000	0.890	1.078	0.373-3.117
	Female	0.08	0.13	1.000	1.000	0.255	0.610	0.259-1.438
rs2363910	All	0.08	0.08	1.000	0.136	0.977	0.989	0.473-2.071
	Male	0.08	0.10	1.000	0.344	1.000	0.859	0.250-2.946
	Female	0.08	0.07	1.000	0.243	0.796	1.136	0.431-2.993

Abbreviations: CI, confidence interval; HWE, Hardy–Weinberg equilibrium; OR, odds ratio; SNP, single nucleotide polymorphisms; SPS, sticky platelet syndrome.

The haplotype analysis showed a significant higher occurrence of 2 haplotypes (aagc: 0.131 vs 0.02, OR: 7.459, 95% CI: 1.604-34.673 in ADRA2A\_4reg haplotype; acg: 0.071 vs 0.000 in ADRA2A\_3reg haplotype), see Table 3. In case we combine haplotype results with SNPs frequency analysis, we find that the most interesting results are results of PEAR1\_H2 haplotype. Using EM algorithm, the estimated minor haplotypes constructed from synonymous variants (rs1874445 and rs2230722) were significantly associated with the given SPS phenotype accompanied by DVT. Patients with AT variant have decrease risk of DVT versus healthy controls (0.071 vs 0.218, OR: 0.276, 95% CI: 0.106-0.718), see Table 3.

**Discussion**

Nowadays, it is widely accepted that the pathogenesis of DVT is multifactorial, in which case a single cause may predispose

to but is not sufficient on its own to trigger thrombosis. Consequently, all risk factors, whether congenital or acquired, are relatively “innocent” when considered alone. However, when an individual inherits 1 or more abnormalities, compounded in many cases by environmental hazards, that person may be propelled over a threshold that precipitates the development of thrombosis. An appropriate analogy is that when “the last drop makes the cup run over.”<sup>19</sup> Sticky platelet syndrome represents an independent risk factor in patients with otherwise unexplained DVT. Platelets, intimately involved in the pathogenesis of DVT, can be activated by a variety of agonists through interactions with specific receptors localized on their membrane.

In our work, we focused on variability of the 7 genes (MRVI1, PEAR1, JAK2, FCER1G, PPBP, ADRA2A, and SHH), and we examined the association between selected polymorphisms and the risk for DVT in patients with SPS type

**Table 3.** Haplotype Frequencies in Patients With SPS and Control Individuals.

Haplotype		Frequency (SPS)	Frequency (Control)	P Value	OR	95% CI
ADRA2A_4reg	gcgc	0.464	0.475	.499	0.957	0.536-1.709
	gcac	0.095	0.149	.193	0.604	0.243-1.502
	gcgt	0.083	0.149	.128	0.521	0.202-1.346
	aagc	0.131	0.020	.003	7.459	1.604-34.673
	gcat	0.024	0.010	.430	2.439	0.217-27.379
	aagt	0.024	0.020	.617	1.207	0.166-8.759
	gagc	0.036	0.010	.245	3.704	0.379-36.286
ADRA2A_3reg	gcg	0.512	0.594	.166	0.717	0.340-1.285
	Gca	0.131	0.218	.090	0.541	0.245-1.193
	Aag	0.167	0.099	.126	1.82	0.763-4.341
	Acg	0.071	0.000	.008	–	–
	Gag	0.010	0.036	.245	3.704	0.378-36.286
PEAR1_H2	AG	0.012	0.000	.454	–	–
	GT	0.024	0.010	.430	2.439	0.217-27.379
	AT	0.071	0.218	.004	0.276	0.106-0.718

Abbreviations: ADRA, alpha2A adrenergic receptor 2A; CI, confidence interval; OR, odds ratio; PEAR, platelet endothelial aggregation receptor 1; SPS, sticky platelet syndrome.

II. According to our knowledge, the influence of our selected SNPs on platelet function in acquired or inherited platelet disorders in patients with SPS manifested as DVT has not been studied and published yet.

The goal of our study was to determine the prevalence of selected SNPs in patients and controls and to analyse the contribution of polymorphisms to the pathogenesis of DVT in patients with platelet hyperaggregability after low concentration of ADP and/or EPI. The target population of our analysis consisted of patients with SPS type II, who develop DVT more often and at younger age than the general population, which draws the attention rather to genetic than to acquired risk factors. We identified 2 protective SNPs within PEAR1 gene and 1 risk SNP within ADRA2A gene (PEAR1: rs12041331; rs12566888; ADRA2A: rs1800545). A haplotype analysis of 4 SNPs within ADRA2A gene identified a risk haplotype aagc ( $P = .003$ ). Moreover, we identified 1 protective haplotype within PEAR1 gene (AT,  $P = .004$ ). This finding suggests that the ADRA2A rs1800545 allele is associated with increased risk of DVT in patients with SPS type II. Moreover, carriership of rs12041331 and rs12566888 localized within PEAR1 gene has protective effect against DVT in patients with SPS type II.

Kotuličová et al reported about significantly higher occurrence of 4 SNPs (rs1671153, rs1654419, rs11669150, and rs1613662) within glycoprotein 6 gene in patients with SPS type II and history of VTE compared to healthy population.<sup>20</sup> In 2012, we pointed out that 3 selected SNPs (rs1671153, rs1654419, and rs1613662) of the GP6 gene occur more frequent in patients with SPS type II with history of miscarriage.<sup>21</sup> In our previous research, we identified 2 SNPs within PEAR1 gene (rs12041331 and rs12566888) with lower occurrence in patients with SPS type II manifested with fetal loss.<sup>22</sup> If we combine these 3 studies, we can see that the SPS is associated with variants localized within GP6 (rs1671153, rs1654419, rs11669150, and rs1613662) and ADRA2A (rs1800545) gene. The presence of 2 SNPs within PEAR1 (rs12041331 and

rs12566888) gene has a protective effect against VTE and fetal loss in patients with SPS type II.

There were several limitations in our study, including the limited number of patients with DVT and a possible bias in patient selection. Sticky platelet syndrome in the Slovak population in association with history of DVT is extremely rare. Despite this, this study represents the largest sample of patients having SPS with history of DVT. As far as the SPS diagnostic process is concerned, there are also several limitations from a methodological standpoint. Platelet aggregability is greatly affected by preanalytical issues, and therefore, interpretation of platelet hyperaggregability is potentially accordingly adversely influenced. Moreover, functional studies of ligands and pathways which are going to analyse different isoforms have to be performed. In addition, this study had not a prospective observational design for new or recurrent VTE events. It is very possible that epigenetics changes or phenotype changes in coagulation parameters that occur after a first VTE might alter the predictability of SNPs for a VTE event.

## Conclusions

This study suggests that ADRA2A allele (rs1800545) might be an independent risk factor, and PEAR1 alleles (rs12041331 and rs12566888) might be an independent protective factor for DVT in patients with SPS type II. These alleles could contribute to the SPS phenotype, although not as a main genetic cause but as an additional genetic defect. Therefore, we assume that the SPS has probably a polygenic mode of inheritance, where each gene locus had an independent effect on a single phenotype.

## Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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