

Species-Specific Regulation of t-PA and PAI-1 Gene Expression in Human and Rat Astrocytes

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ABSTRACT: In recent years, the role and physiological regulation of the serine protease tissue-type plasminogen activator (t-PA) and its inhibitors, including plasminogen activator inhibitor type-1 (PAI-1), in the brain have received much attention. However, as studies focusing these issues are difficult to perform in humans, a great majority of the studies conducted to date have utilized rodent *in vivo* and/or *in vitro* models. In view of the species-specific structural differences present in both the t-PA and the PAI-1 promoters, we have compared the response of these genes in astrocytes of rat and human origin. We reveal marked quantitative and qualitative species-specific differences in gene induction following treatment with various physiological and pathological stimuli. Thus, our findings are of importance for the interpretation of previous and future results related to t-PA and PAI-1 expression.

KEY WORDS: astrocytes, tissue-type plasminogen activator, plasminogen activator inhibitor type-1, gene expression, species-specific

CITATION: Tjärnlund-Wolf et al. Species-Specific Regulation of t-PA and PAI-1 Gene Expression in Human and Rat Astrocytes. *Gene Regulation and Systems Biology* 2014;8:113–118 doi: 10.4137/GRSB.S13387.

RECEIVED: October 9, 2013. **RESUBMITTED:** February 17, 2014. **ACCEPTED FOR PUBLICATION:** February 25, 2014.

ACADEMIC EDITOR: James Willey, Editor in Chief

TYPE: Short Report

FUNDING: The present study was supported by the Swedish Research Council, grants from the Swedish State, the Swedish Heart-Lung, Swedish Brain, Yngve Land, Rune and Ulla Amlöv, Åke Wiberg, Magnus Bergvall, and Edit Jacobson Foundations.

COMPETING INTERESTS: Authors disclose no potential conflicts of interest.

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Introduction

Tissue-type plasminogen activator (t-PA) converts plasminogen into plasmin, which in turn dissolves the fibrin structure of thrombi. The fibrinolytic properties of t-PA have been widely studied, but it has become evident that the role for t-PA in the brain extends beyond that of regulating vascular patency. In the brain, t-PA is produced by neurons and glial cells¹ and is involved in physiological and pathophysiological processes, including various aspects of synaptic plasticity and neurodegeneration.^{2–4}

In the context of ischemic stroke, aside from the beneficial effects of t-PA as a thrombolytic, much attention has been drawn to its proposed neurotoxic effects. Following excessive neuronal depolarization or glial activation, t-PA accumulates in the extracellular space. This may result in neurotoxicity

and initiation of deleterious proteolytic cascades.^{1,3} Indeed, endogenous t-PA has been shown to potentiate the extent of ischemic injury,^{5,6} while astrocyte-derived PAI-1 can rescue neurons from these effects.⁷

An understanding of the role and regulation of t-PA and its inhibitors in the CNS may provide crucial tools for the development of novel neuroprotective strategies. However, as studies addressing these issues are difficult to perform in humans, a great majority of the studies conducted to date have utilized *in vivo* and *in vitro* animal models. In view of species-specific structural differences between the t-PA and PAI-1 promoters, the question remains as to how pertinent the results obtained in these animal models can be translated into the human brain. In an attempt to examine this issue, we have compared the effects of physiological and pathophysiological stimuli, as



well as activators of protein kinases, on the expression of the t-PA and PAI-1 genes in astrocytes of human and rat origin.

Methods

Cell culture. Human astrocytes (Clonetics, Walkersville, MD) were cultured in astrocyte growth medium (AGM) supplemented with 3% fetal bovine serum (FBS) as described.⁸ All experimental series were performed using astrocytes from two to three individuals. Primary cortical rat astrocytes were prepared from newborn (P1–P2) Sprague-Dawley rats according to protocols approved by the Ethics Committee of the University of Gothenburg and cultured as described.⁹ Human and rat astrocyte cultures were >95% glial fibrillary acidic protein (GFAP) positive.

Gene expression studies. Human or rat astrocytes were seeded in 12-well dishes and allowed to reach 100% confluency. Cells were incubated in culture medium containing retinoic acid (RA, 10^{-6} M), phorbol 12-myristate 13-acetate (PMA, 10^{-7} M), forskolin (2×10^{-5} M), interleukin-6 (IL-6, 10 ng/mL), tumor necrosis factor- α (TNF- α , 5 ng/mL), transforming growth factor- β (TGF- β , 1 ng/mL), or dimethyl sulfoxide (DMSO) vehicle (0.2%) for the indicated time periods. For oxygen glucose deprivation (OGD), cells were transferred to a hypoxic chamber (BioSpherix, Redfield, NY) and maintained under hypoxic conditions (1% O₂) in serum-free and glucose-free Dulbecco modified Eagle medium (DMEM). Control cultures were maintained under normal culturing conditions in serum-free DMEM containing glucose. Three treatment series were performed on two separate occasions ($n = 6$).

t-PA and PAI-1 mRNA levels. Following treatment, cells were lysed with lysis buffer (Buffer RLT; QIAGEN, Hilden, Germany), and lysates were stored at -70°C . Total cellular RNA was isolated using an RNeasy mini kit (QIAGEN) and mRNA was converted to cDNA with the GeneAmp RNA PCR kit (Applied Biosystems, Foster City, CA). Target mRNA was quantified by TaqMan real-time RT-PCR and normalized relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA or 18S, for OGD experiments, according to a standardized protocol.^{8,10,11} The oligonucleotide primers and TaqMan probes for quantification of human t-PA, PAI-1, GAPDH, and 18S mRNA were as follows: t-PA forward primer (fp) 5'-GGCCTTGTCTCCTTTCTATTTCG-3', t-PA reverse primer (rp) 5'-AGCGGCTGGATGGGTACAG-3'; t-PA probe (pr) 5'-(FAM)TGACATGAGCCTCCTTCAGCCGCT(TAMRA)-3'; PAI-1 (fp) 5'-GGCTGACTTCACGAGTCTTTCA-3'; PAI-1 (rp) 5'-TTCACCTTCTGCAGCGCCT-30, PAI-1 (pr) 5'-(FAM)ACCAAGAGCCTCTCCACGTCGCG(TAMRA)-3'; GAPDH (fp) 5'-CCACATCGCTCAGACACCAT-3', GAPDH (rp) 5'-CCAGGCGCCCAATACG-3'; GAPDH (pr) 5'-(FAM)AAGGTGAAGGTTCGAGTCAACGGATTTG(TAMRA)-3'; and Applied Biosystems Assay-on-Demand Hs99999901_s1. For amplification of rat t-PA, PAI-1, GAPDH, and 18S Applied Biosystems Assay-on-Demand Rn01482584_m1, Rn01481341_m1, Rn01462662_g1,

and TaqMan, Eukaryotic 18S rRNA Endogenous Control was used.

Statistical analysis. Values are presented as mean and standard error of the mean (SEM), and a P -value of 0.05 or less was considered statistically significant. Differences in the mRNA levels between human and rat cultures were evaluated by the unpaired Student's t -test (SPSS Inc., Chicago, IL).

Results and Discussion

With regard to t-PA, the most pronounced difference between human and rat astrocytes was observed in response to treatment with the protein kinase A (PKA) activator forskolin (Table 1A). In human cells, forskolin caused more than a 50% reduction in t-PA expression compared to control-treated cells, whereas t-PA expression was strongly (>4-fold) induced in rat astrocytes. Species-specific differences in t-PA response following cAMP activation have also been described for other cell types.¹² We have shown that the protein kinase C (PKC) activator PMA and RA induce t-PA expression in human astrocytes⁸ (data included in Table 1A for comparative purposes). In rat astrocytes, t-PA expression in response to these stimuli was markedly different as t-PA mRNA levels were only weakly increased or even down-regulated following treatment with PMA or RA (Table 1A).

With regard to PAI-1, PKC activation resulted in the most pronounced difference, where prolonged PMA treatment (20 hours) induced a 14-fold induction in rat PAI-1 expression, while human PAI-1 levels were unchanged (Table 1B). Significant differences between human and rat PAI-1 induction were also observed following prolonged OGD (Table 1B). An additional series of experiments were performed with cytokines (human TGF- β , TNF- α , and IL-6), all of which resulted in significant species-specific differences in t-PA and PAI-1, eg, 20 hours treatment with TGF- β resulted in a 30% t-PA mRNA reduction in human cells and a 4-fold induction in rat cells (Table 1A), whereas 20 hours treatment with TNF- α reduced human PAI-1 expression and enhanced rat PAI-1 expression 4-fold (Table 1B). Our findings are in agreement with studies on other cell types showing that PAI-1 regulation in response to cytokines and hypoxia is highly complex, involving different gene regulatory elements and transcription factors depending on cell type and/or species.¹³

A potential mechanism behind species-specific differences in t-PA and PAI-1 gene expression is DNA sequence variations. Indeed, the rat t-PA promoter contains a perfect cAMP-responsive element (CRE) consensus sequence (TGACGTCA), whereas the human and mouse counterparts contain a single nucleotide substitution in the core of the motif (G→A, Figure 1A), potentially affecting both transcriptional responsiveness to cAMP-elevating agents and CRE-binding protein (CREB) binding affinity.^{12,14} In line with this, we observed a strong induction of rat t-PA and a reduced expression of human t-PA following treatment with forskolin. As several studies show that this CRE-like site is a key DNA element mediating human t-PA gene transcription

Table 1. t-PA and PAI-1 mRNA expression in human and rat astrocytes following treatment.

DURATION OF TREATMENT	3 H			20 H			
	SPECIES	HUMAN	RAT	P-VALUE, T-TEST HUMAN VS. RAT	HUMAN	RAT	P-VALUE, T-TEST HUMAN VS. RAT
A. Fold increase in t-PA mRNA in human and rat astrocytes, mean (SEM)							
PMA		3.38 (0.19)	1.57 (0.09)	< 0.001	4.71 (0.36)	1.57 (0.07)	< 0.001
Forskolin		0.49 (0.02)	4.60 (0.39)	< 0.001	0.39 (0.06)	1.47 (0.10)	< 0.001
RA		1.67 (0.09)	0.85 (0.04)	< 0.001	4.30 (0.54)	1.38 (0.06)	< 0.001
TGF- β		1.52 (0.04)	1.98 (0.08)	< 0.001	0.70 (0.04)	3.88 (0.14)	< 0.001
TNF- α		2.30 (0.18)	1.59 (0.06)	< 0.01	0.81 (0.05)	1.19 (0.05)	< 0.001
IL-6		1.25 (0.05)	2.32 (0.14)	< 0.001	0.91 (0.03)	1.56 (0.06)	< 0.001
OGD		1.07 (0.06)	1.08 (0.10)	NS	0.66 (0.21)	0.61 (0.14)	NS
B. Fold increase in PAI-1 mRNA in human and rat astrocytes, mean (SEM)							
PMA		1.41 (0.07)	1.81 (0.07)	NS	1.01 (0.09)	13.99 (0.71)	< 0.001
Forskolin		0.81 (0.06)	0.22 (0.03)	< 0.001	0.29 (0.05)	0.56 (0.06)	< 0.01
RA		1.26 (0.10)	0.80 (0.02)	< 0.01	0.98 (0.05)	1.48 (0.06)	< 0.001
TGF- β		1.98 (0.11)	2.67 (0.10)	< 0.01	1.10 (0.10)	1.35 (0.05)	< 0.05
TNF- α		2.08 (0.11)	1.39 (0.05)	< 0.01	0.82 (0.04)	3.54 (0.12)	< 0.001
IL-6		1.76 (0.05)	0.68 (0.03)	< 0.001	1.28 (0.04)	0.72 (0.07)	< 0.001
OGD		1.59 (0.12)	1.55 (0.19)	NS	0.72 (0.12)	2.63 (0.20)	< 0.001

Notes: Human and rat astrocyte cultures were treated with PMA (10^{-7} M), forskolin (2×10^{-5} M), RA (10^{-6} M), TGF- β (1 ng/mL), TNF- α (5 ng/mL), IL-6 (10 ng/mL), OGD, or vehicle for the indicated time periods. Total cellular RNA was extracted, and mRNA was converted into cDNA. Target mRNA (cDNA) was quantified by real-time RT-PCR and normalized relative to GAPDH or 18S (for OGD) mRNA. Results are expressed as fold increase compared with control cultures. Each data point represents the average of three treatment series performed on two separate occasions ($n = 6$). Each sample was analyzed in triplicate for target and control gene. Differences in the mRNA levels between human and rat cultures were evaluated by the unpaired Student's *t*-test. NS indicates not significant. The results for human t-PA and PAI-1 following RA and PMA treatments and human PAI-1 after treatment with TGF- β , TNF- α , and IL-6 were reproduced from Refs. 8 and 10, respectively.

following PKC activation,^{14–17} it is also possible that the sequence variability at this site contributes to the differential levels of t-PA gene activation detected in the human and rat astrocytes. The observed opposite species-specific responses following PKA and PKC activation may seem contradictory given that both PKA and PKC activators act through the CRE, yet PKA treatment induced high expression of rat t-PA and reduced human t-PA whereas PKC treatment produces the opposite effect. However, it should be noted that the one base difference in the core of the human CRE-like element changes it to another transcription factor consensus sequence specifically recognized by AP1,¹⁸ a *trans*-acting factor mediating PKC-induced gene expression. In line with this, studies on human endothelial and HeLa cells show that treatment with PMA results in a selective recruitment of AP1 family members *fos* and *jun* to the CRE-like site.^{15,16} Thus, while the rat CRE, as opposed to that of the human CRE-like site, provides a high affinity binding site for CREB, it is possible that this single nucleotide substitution in the human element renders this site more responsive to PKC-mediated transactivation. If so, this could provide a plausible explanation behind the higher level of t-PA expression observed in human astrocytes compared to that of the rat following treatment with PMA. In this respect, studies addressing

cell type-specific patterns of t-PA gene regulation following PMA treatment show that differences in the binding affinity of individual transcription factors associated with the human CRE-like site correlate with the degree of PMA-induced t-PA promoter activation.^{15,17} It is also of interest to note that a suppression of t-PA in HT1080 cells mediated by PMA was associated with binding of CREB-1 to the CRE-like site.¹⁷ In addition, functional analyses of the human t-PA promoter show that the GC-boxes located in the proximal part of the promoter, particularly GC-box III, are important for PMA-induced t-PA transcription,^{14–16} and a possible involvement of the CCAAT-binding transcription factor/nuclear factor-1 (CTF/NF-1) site cannot be excluded.¹⁵ Whether the species-specific sequence dissimilarities at these sites affect transcription factor binding affinity and t-PA promoter inducibility remains to be determined.

In terms of PAI-1, the strikingly different level of induction observed in human versus rat cells following PMA treatment was unexpected. Previous studies show that the transcriptional PAI-1 response to PMA may be mediated via AP1 and Sp1 sites in the proximal promoter (Fig. 1B).^{19,20} Given the high degree of sequence conservation in these regions, it seems likely that additional regulatory elements are involved in this response, and this remains to be explored. In

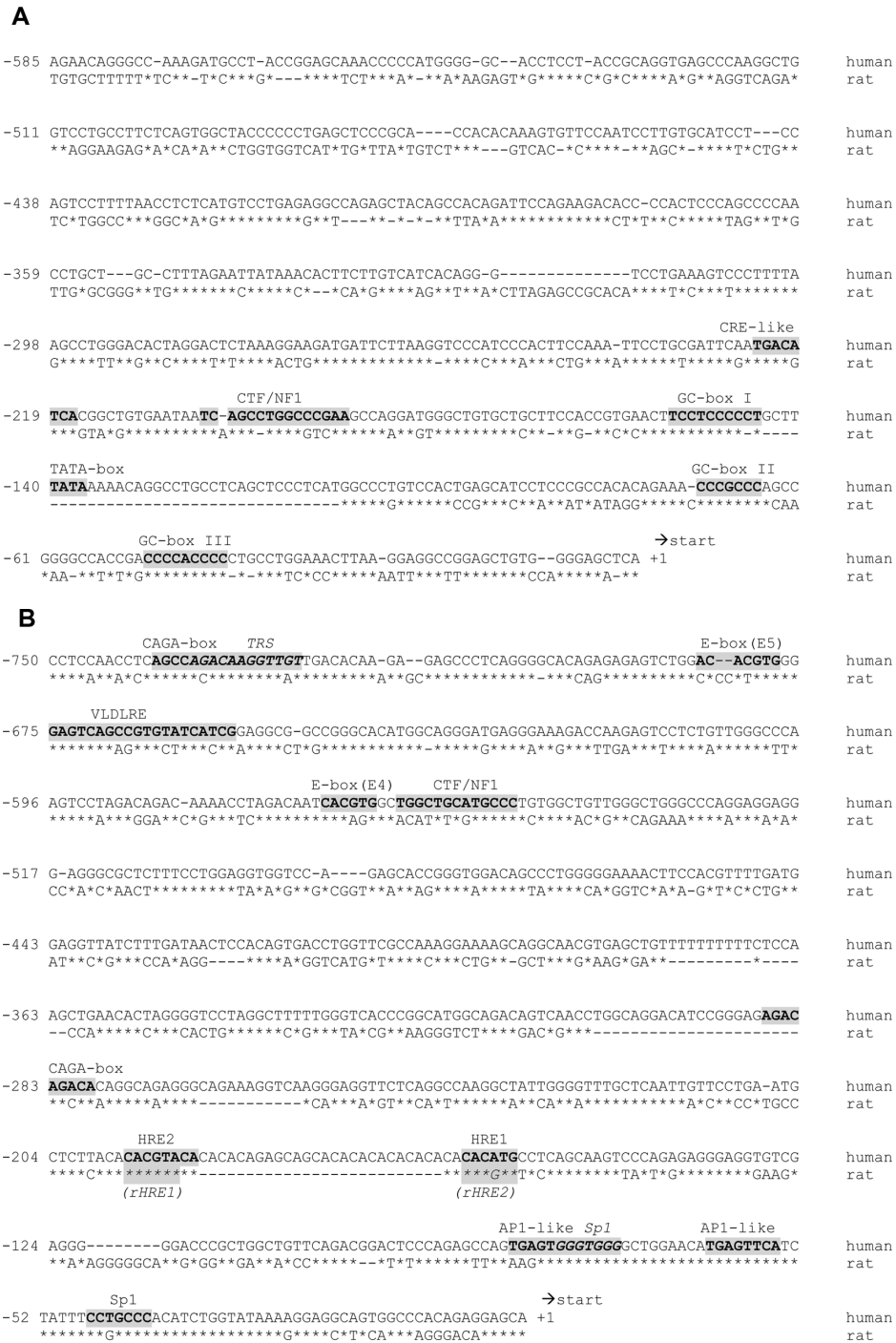


Figure 1. Alignment of the t-PA and PAI-1 human and rat promoter regions.

Notes: The rat t-PA and PAI-1 promoters were aligned to the corresponding human –585 bp t-PA (A) and –750 bp PAI-1 (B) promoters, respectively, using the Jotun-Hein clustal alignment algorithm (MegAlign program, DNASTAR). The t-PA and PAI-1 promoters are numbered relative to the respective transcription start sites. *denotes identical nucleotides, letters indicate substitutions, and dashes represent gaps introduced into the sequence to obtain optimal homology alignment. Transcription factor binding sites are highlighted in gray and with bold nucleotides.

Abbreviations: CRE, cAMP-responsive element; Sp1, stimulatory protein-1 response element; CTF/NF-1, CCAAT-binding transcription factor/nuclear factor-1 site; CAGA-box, Smad 3 and 4-binding element; HRE, hypoxia-responsive element; rHRE, rat hypoxia-responsive element; E-box, enhancer box; AP1-like, activator protein-1-like binding element; TRS, TGF-β responsive sequence; VLDLRE, very low density lipoprotein responsive element.

this context, it should also be noted that our real-time RT-PCR data do not necessarily reflect changes in gene transcription. Thus, it is also possible that additional regulatory control may be exerted post-transcriptionally, for instance at the level

of mRNA stability, and this may also be subject to species variation. A significant difference in PAI-1 expression was also observed following prolonged OGD treatment (2.6-fold induction of rat PAI-1 and 28% reduction in human PAI-1,

Table 1B). Our results are supported by other studies showing species-specific effects in PAI-1 gene regulation and induction following hypoxia. While rat PAI-1 is induced at very mild levels of hypoxia (8% O₂),²¹ severe hypoxia (<2% O₂) was required to induce human PAI-1 expression.²² The *trans*- and *cis*-acting factors involved in the PAI-1 response to hypoxia have been extensively studied. Collectively, data suggest a highly complex regulatory pattern, which is dependent on not only species but also cell type, hypoxia level, and context.

Hypoxia-inducible factor-1 (HIF-1) is a central *trans*-acting factor conveying hypoxia-mediated PAI-1 induction, but several other important factors have been identified, such as USF-2, HIF-2, EGR-1, and Net (reviewed by Dimova and Kietzmann¹³). Both the human and rat PAI-1 promoters contain several potential hypoxia-responsive elements (HREs). While the gene regulatory mechanisms conveying the response of the PAI-1 gene to hypoxia in astrocytes remain to be established, studies on other human cell types suggest an involvement of several *cis*-elements, particularly HRE2 and two distally located E-boxes (E4 and E5).^{22,23} These two E-boxes are not present in the rat promoter (Fig. 1B). In rat cells, the two main gene regulatory elements conveying the hypoxia response appear to be two HREs: rHRE1 and rHRE2,^{21,24} the later of which shares almost perfect sequence homology with the human HRE1 (Fig. 1B). However, because of the one base substitution in the core of the human motif, the human HRE1 lacks binding of hypoxia-inducible factors and is unable to convey hypoxia-induced promoter activation.²² In this context, it should also be noted that HIF-1 may be activated by other stimuli, such as hormones and cytokines.²⁵ Thus, species-specific differences in the elements binding HIF-1 may also affect PAI-1 gene responses under normoxic conditions. Furthermore, marked quantitative and qualitative differences in the *trans*-acting factors that mediate the PAI-1 response to hypoxia have been described, and shown to be dependent on both cell type and species.^{21–24} It should be added that in addition to the possibility of species-specific changes in post-transcriptional regulation, there may also be differential epigenetic mechanisms controlling the regulation of these genes but this is yet to be explored.

In summary, the exact mechanisms behind the observed species-specific differences in the response to the factors assayed in the present report remain to be established, but the clear sequence variation in important transcription factor binding sites (Fig. 1) is likely to be a key contributing component. Other mechanisms may include species-specific differences in the levels of transcription factors, co-factors, and second messenger systems involved in signal transduction, as well as epigenetic mechanisms. Another consideration relates to species-specific post-transcriptional regulatory events controlling t-PA and PAI-1 gene expressions, ie, changes in mRNA stability that are well known to be influenced by changes in sequence of the transcript itself, mostly in the 3'-UTR. While this was not specifically evaluated in this study, the fact that

we have identified marked species-specific changes in t-PA and PAI-1 gene expression at the mRNA level in response to various agonists underlies the difficulties in interpreting results from rodents to humans.

Conclusion

There are marked quantitative and qualitative species-specific differences in the responses of the t-PA and PAI-1 genes in astrocytes. Whether the same is true for other species, including mouse, remains to be established. In view of the present data, although the use of rodent models may provide helpful research tools for scientists seeking mechanisms behind disease and potential targets for future therapies, extrapolations to the *in vivo* situation in humans need to be made with caution.

Acknowledgements

We would like to thank the SWEGENE Göteborg Genomics Core Facility platform, which was funded by a grant from the Knut and Alice Wallenberg Foundation.

Author Contributions

AT-W, KH, and CJ conceived and designed the experiments. AT-W and KH analyzed the data. AT-W wrote the first draft of the manuscript. KH, CJ, and RLM contributed to the writing of the manuscript. All authors agreed with manuscript results and conclusions. AT-W, KH, CJ, and RLM jointly developed the structure and arguments for the paper. AT-W, KH, FB, MN, RLM and CJ made critical revisions, and all of them reviewed and approved the final manuscript.

DISCLOSURES AND ETHICS

As a requirement of publication the authors have provided signed confirmation of their compliance with ethical and legal obligations including but not limited to compliance with ICMJE authorship and competing interests guidelines, that the article is neither under consideration for publication nor published elsewhere, of their compliance with legal and ethical guidelines concerning human and animal research participants (if applicable), and that permission has been obtained for reproduction of any copyrighted material. This article was subject to blind, independent, expert peer review. The reviewers reported no competing interests.

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