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SHORT COMMUNICATION

Single-run chemo- and enantio-selective high-performance liquid chromatography separation of tramadol and its principal metabolite, O-desmethyltramadol, using a chlorinated immobilized amylose-based chiral stationary phase under multimodal elution conditions

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A direct enantio- and chemo-selective high-performance liquid chromatography method was developed for separating the enantiomers of the pain medicine tramadol and its O-desmethyl active metabolite in a single run. The simultaneous separation was achieved on the immobilized-type amylose tris(3-chloro-5-methylphenylcarbamate) chiral stationary phase. The method was optimized in normal-phase and reversed-phase using ethanol as an organic modifier. It was demonstrated that ethanol is a valid alternative to the predominant high-performance liquid chromatography solvents in use for the preparation of reversed-phase eluents such as methanol and acetonitrile. With the green mobile phase ethanol-water-diethylamine, 80:20:0.1 (v/v/v) the limits of quantification for the (+)/(-)-enantiomers of tramadol and its O-desmethyl active metabolite were 1.14/1.16 and 1.33/1.40 μg mL^{-1}, respectively.

KEYWORDS
amylose tris(3-chloro-5-methylphenylcarbamate), enantioseparation, green analysis, high-performance liquid chromatography, O-desmethyltramadol, tramadol

1 | INTRODUCTION

Tramadol, (TMD) (2-dimethylamino methyl)-1-(3′-methoxyphenyl)cyclohexanol hydrochloride) is a 4-phenyl-piperidine derivative correlated to the codeine that is worldwide used to treat both severe pains after surgery or trauma, and chronic cancer/non-cancer pain [1].

Although TMD has two stereogenic centers in the cyclohexane ring (Figure 1), the commercially available drug products contain only the racemic mixture of the (1*R*,2*R*) and (1*S*,2*S*) enantiomers (also designated as (+) and (-) enantiomers) of *cis*-(2-dimethylamino methyl)-1-(3-methoxyphenyl) cyclohexanol.

TMD exerts analgesic effects via a multimodal mechanism of action. The non-opioid component is either due to the (+)-enantiomer of TMD which contributes to analgesia by inhibiting the reuptake of serotonin, or the (-)-enantiomer which inhibits the reuptake of noradrenaline [2]. The O-desmethyl active metabolite (M1) of TMD (Figure 1), on the other hand, acts on the μ-opioid

Abbreviations: ACMPC, amylose tris(3-chloro-5-methylphenylcarbamate); CSP, chiral stationary phase; CYP2D6, cytochrome P450 2D6; DCM, dichloromethane; DEA, diethylamine; EA, ethyl acetate; IPA, 2-propanol; M1, O-desmethyltramadol; MTHF, 2-methyltetrahydrofuran; TMD, tramadol

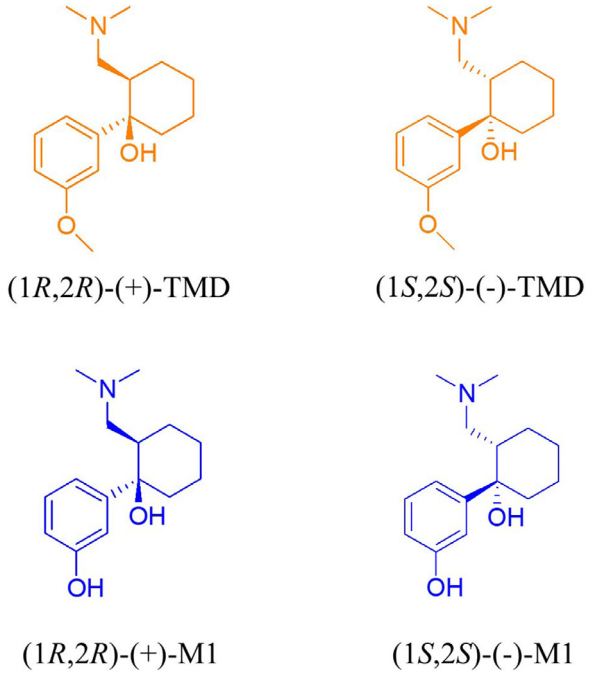

FIGURE 1 Structures of the enantiomers of tramadol and O-desmethyltramadol

receptor. The affinity of M1 for the μ-opioid receptor is only due to the (*R*)-(+)-enantiomer and it is about one-tenth that of morphine and 700 times higher than TMD [3].

O-Demethylation of TMD is catalyzed primarily by the hepatic cytochrome P450 2D6 (CYP2D6) isoform. As this enzyme shows genetic polymorphism, patients with CYP2D6 ultrarapid can produce relatively high and dangerous plasma concentrations of (+)-M1, whereas CYP2D6 poor metabolizers have relatively low plasma concentrations of the active metabolite [4].

Thus, M1, and in particular, its dextrorotatory enantiomer, could provide analgesic benefits similar to TMD but with a comparatively more favorable safety profile [5].

The most convenient analytical approach to determine the (+)/(−)-enantiomer ratios of TMD and its active metabolite M1 after oral administration of racemic TMD is to use enantio- and chemo-selective high-performance liquid chromatography (HPLC) conditions. Up to now this protocol, which is named the single-run approach because it requires the use of a single highly selective and efficient chiral chromatographic column [6], has been successfully applied to the racemic compounds TMD and M1 only in a few works [7–9]. Under normal-phase mode, the separation of the enantiomers of TMD and M1 was achieved on the coated-type amylose-based Chiralpak AD chiral stationary phase (CSP) [8,9]. At 30°C, the retention times were: (+)-TMD 7.9 min, (-)-TMD 9.1 min, (+)-M1 17.9 min and (-)-M1 19.1 min [9]. A reversed-phase method was developed using an α_1-acid glycoprotein-based CSP with a mobile phase consisting of 30 mM diammonium hydrogen phosphate buffer–acetonitrile–triethylamine (98.9:1:0.1, v/v/v), adjusted to pH 7 by phosphoric acid [7]. The enantiomers of two samples were eluted within 32 min.

Considering what mentioned above and our continuing interest in the development of novel chiral HPLC methods for compounds of pharmaceutical interest, the present study aimed to investigate the ability of the immobilized-type amylose-based Chiralpak IG-3 CSP to simultaneously separate the two couple of enantiomers of TMD and M1 under normal- and reversed-phase conditions. To achieve this goal, different parameters such as column temperature, mobile phase composition, and flow rate were carefully evaluated.

2 | MATERIALS AND METHODS

2.1 | Reagents and chemicals

Diethylamine (DEA) and TMD hydrochloride were obtained from Aldrich (Milan, Italy). M1 was synthesized starting from TMD as reported previously [10]. HPLC-grade solvents *n*-hexane, ethanol, 2-propanol (IPA), dichloromethane (DCM), ethyl acetate (EA), and 2-methyltetrahydrofuran (MTHF) were obtained from Aldrich (Milan, Italy) and filtered (0.22 μm filter) before use. HPLC analyses were carried out on the Chiralpak IG-3 (250 mm x 4.6 mm, 3 μm), Chiralpak AD (250 mm x 4.6 mm, 10 μm) and Chiralcel OD (250 mm x 4.6 mm, 10 μm) columns (Chiral Technologies Europe, Illkirch-Graffenstaden, France).

2.2 | Instruments

Chromatographic experiments were performed on the UHPLC Jasco LC-4000 (Jasco, Tokyo, Japan). This instrument included a binary pumping system with a maximum flow rate of 2 mL min^{-1}, an auto-sampler with an injection loop volume of 50 μL (used in partial loop mode), an MD-4010 photodiode array detector with a 2.7 μL internal volume flow cell, and a column oven. Data acquisition, data handling, and instrument control were performed by Jasco ChomNAV software.

To establish the absolute configuration and enantiomeric elution order, enantioenriched forms of TMD and M1 were isolated by repetitive enantioseparations on the 250 mm x 4.6 mm Chiralcel IG-3 column (mobile phase: *n*-hexane-ethanol-DEA 100:5:0.1 (v/v/v); column temperature: 25°C) Next, a racemic sample of test compounds was spiked with the second eluted enantiomer

and, successively, analyzed on the 250 mm x 4.6 mm Chiralpak AD and 250 mm x 4.6 mm Chiralcel OD columns using the eluent conditions reported by Elsing and Blaschke [11]. As previously reported, (+)-TMD and (+)-M1 are eluted before the (-) enantiomers. The same enantiomer elution order was observed using the mobile phase *n*-hexane-ethanol-DEA 100:5:0.1 (v/v/v).

2.3 | HPLC operating conditions

Fresh standard solutions of chiral samples were prepared by dissolving 0.25 mg of racemates in 1 mL of the mobile phase. The injection volume was 20 μL. Solvents and samples were filtered through 0.22 μm filters. The hold-up time was estimated by using 1,3,5-tri-*tert*-butylbenzene as a marker and pure ethanol as a mobile phase.

The linearity was evaluated using standard solutions at concentrations of racemic samples ranging from 0.28 mg mL^{-1} to 2.8 μg mL^{-1}. The concentrations of the solutions were plotted against the corresponding peak area responses of the enantiomers of TMD and M1 free bases, and linear regression equations were then calculated. The limit of quantitation (LOQ) parameter represents the concentration of analyte that would yield S/N ratios of 10, according to the European Pharmacopeia guidelines.

3 | RESULTS AND DISCUSSION

3.1 | Enantioseparation of TMD and M1 under normal-phase conditions

The immobilized polysaccharide-based Chiralpak IG-3 CSP consists of amylose tris(3-chloro-5-methylphenylcarbamate) (ACMPC) immobilized onto 3 μm silica particles. The meta-substituted ACMPC-based CSPs have shown a great variety of enantio- and chemoselectivity under both normal-phase and reversed-phase elution modes [6, 12–15].

To develop an effective chromatographic method able to resolve the racemic TMD and M1 in a single run, normal-phase mixtures *n*-hexane-ethanol-DEA 100:10:0.1 (v/v/v) and *n*-hexane-IPA-DEA 100:10:0.1 (v/v/v) were initially tested as mobile phases in combination with the 250 mm x 4.6 mm Chiralpak IG-3 column. The chromatographic parameters obtained at 25°C are shown in Table 1.

A substantial change in enantio- and chemo-selectivity resulting from the employed alcohol modifier was observed. With IPA, the enantiomers of TMD were baseline resolved (entry 2, $\alpha = 1.54$, Rs = 6.95) but those of M1 were just weakly separated (entry 2, $\alpha = 1.04$, Rs < 1). In addition, coelution of the second eluted enantiomer of TMD and the first eluted enantiomer of M1 (this critical pair is named TMD*/M1*) occurred. On the contrary, using ethanol as an alcohol mobile phase modifier, enantioseparation and resolution increased for M1 (entry 1, from 1.04 to 1.20) and decreased for TMD (entry 2, from 1.54 to 1.21). Under this elution condition, the chemoselectivity exhibited by the Chiralpak IG-3 CSP remained low again (Rs < 1). It is worth mentioning that a reversed enantiomer elution sequence of M1 was obtained as the alcohol modifier changed (with ethanol (*R*)-(+)-M1 eluted before (*S*)-(-)-M1). Besides the alcohol components, in normal-phase mode other solvents (named non-standard solvents) are also frequently used to modify the selectivity of the immobilized-type polysaccharide-based CSPs [16]. Data summarized in Table 1 show the effects on the chromatographic performance of the Chiralpak IG-3 CSP caused by the replacement of five volumes of alcohol with the same volume of DCM, EA, or MTHF (entries 3–8). Regarding the enantioselectivity, no substantial enhancement was registered for both test compounds whereas a moderate enhancement in the chemoselectivity was observed (entries 3, 4, 7, and 8).

Keeping in mind the key role played by temperature on retention and selectivity [17], a variable temperature study was carried out in the temperature range 15–35°C selecting the mixtures *n*-hexane-ethanol-DEA 100:10:0.1 (v/v/v) and hexane-ethanol-DEA 100:5:0.1 (v/v/v) as mobile phases. The chromatograms obtained through 5°C increments analyzing a sample containing an equimolar concentration of TMD and M1 are shown in Figure 2. In both modalities: i) the retention and enantioselectivity decreased with increasing temperature, ii) a complete and simultaneous separation of two pairs of enantiomers was achieved, and iii) the (*R*)-(+)-enantiomers eluted before (*S*)-(-)-enantiomers. It should be highlighted that with the mobile phase hexane-ethanol-DEA 100:5:0.1 (v/v/v) the resolution of two racemic samples and the critical pair TMD*/M1* was at least 3.17 in the temperature range investigated. Differently, the chemoselectivity of the Chirapak IG-3 CSP in presence of the eluent with higher ethanol content was deeply conditioned by temperature. As shown in Figure 2, increasing the column temperature from 15 to 25°C the sequence of the elution of the critical pair reversed yielding a partial peak overlapping. However, with the temperature above 25°C, the resolution got back to being complete yet.

3.2 | Chemo- and enantioselective HPLC analysis of TMD and M1 under green conditions

To explore the chiral ability of the Chiralpak IG-3 CSP in green elution mode, an analytical strategy based on

TABLE 1 Chromatographic results in normal-phase conditions

Entry	Mobile phase	k_{1TMD}	k_{1M1}	α_{TMD}	α_{M1}	α *[1]	Rs_{TMD}	Rs_{M1}	Rs*[1]
1	*n*-Hexane-EtOH-DEA 100:10:0.1	1.27	1.59	1.21	1.20	1.04	2.60	2.40	<1
2	*n*-Hexane-IPA-DEA 100:10:0.1	1.39	2.13	1.54	1.04	1.00	6.95	<1	NR
3	*n*-Hexane-EtOH-MTHF-DEA 100:5:5:0.1	1.15	1.96	1.21	1.10	1.41	2.31	1.17	4.42
4	*n*-Hexane-EtOH-EA-DEA 100:5:5:0.1	1.23	2.07	1.19	1.15	1.42	2.24	2.01	4.82
5	*n*-Hexane-EtOH-DCM-DEA 100:5:5:0.1	1.28	2.37	1.21	1.06	1.53	2.64	0.85	6.35
6	*n*-Hexane-IPA-MTHF-DEA 100:5:5:0.1	1.37	2.37	1.39	1.05	1.24	5.06	NR	<1
7	*n*-Hexane-IPA-EA-DEA 100:5:5:0.1	1.37	2.68	1.36	1.00	1.44	4.79	NR	5.28
8	*n*-Hexane-IPA-DCM-DEA 100:5:5:0.1	1.26	2.23	1.38	1.00	1.29	5.40	NR	3.39

[1]Enantioseparation and resolution factors of the critical pair second eluted enantiomer of TMD/first eluted enantiomer of M1.
Chromatographic conditions: column, Chiralpak IG-3 (250 mm x 4.6 mm, 3 μm); flow rate, 1 ml min^{-1}; temperature, 25°C; detection, UV at 270 nm. Abbreviations: DCM: dichloromethane; DEA, diethylamine; EA: ethyl acetate; EtOH: ethanol; IPA: 2-propanol; MTHF: 2-methyltetrahydrofuran; NR: not resolved; TMD, tramadol.

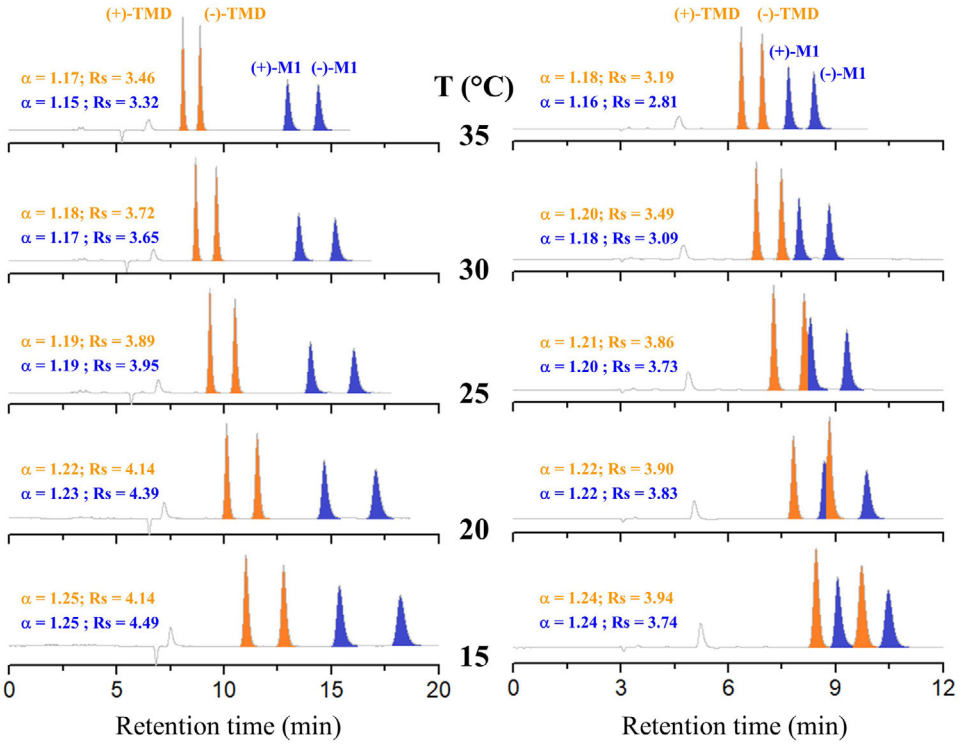

FIGURE 2 Variable-temperature high-performance liquid chromatography (HPLC) single-run resolution of tramadol and O-desmethyltramadol using *n*-hexane-ethanol-diethylamine (DEA) 100:5:0.1 (v/v/v) (left side) and *n*-hexane-ethanol-DEA 100:10:0.1 (v/v/v) (right side) as mobile phases. Chromatographic conditions: column, Chiralpak IG-3 250 mm × 4.6 mm^{-}; flow-rate, 1.0 mL min^{-1}; temperature, from 15 to 35°C; detection, UV at 270 nm

ethanol as an organic modifier and gradual, step-by-step, changes in chromatographic conditions was developed [18]. As reported in Figure 3, under the polar organic mobile phase ethanol/DEA 100/0.1 a good enantioseparation of M1 and the critical pair TRMD*/M1* was achieved (Rs > 1.73). On the other hand, the resolution between the enantiomers of TMD was only 0.67. By keeping the column temperature at 25°C and decreasing the flow rate from 0.5 to 0.3 mL min^{-1}, the resolution factor value of TMD slightly increased to 0.93. In the successive step, the column temperature has decreased to 15°C. Further decreases in temperature improved the resolution factor of TMD (from 0.93 to 1.18) and M1 (from 2.08 to 2.60). As a final step of the optimization protocol, water was added to ethanol/DEA until it yielded 20%. Under reversed-phase conditions, the test compounds were satisfying resolved (Rs > 1.77), without increasing dramatically the analysis time (<30 min).

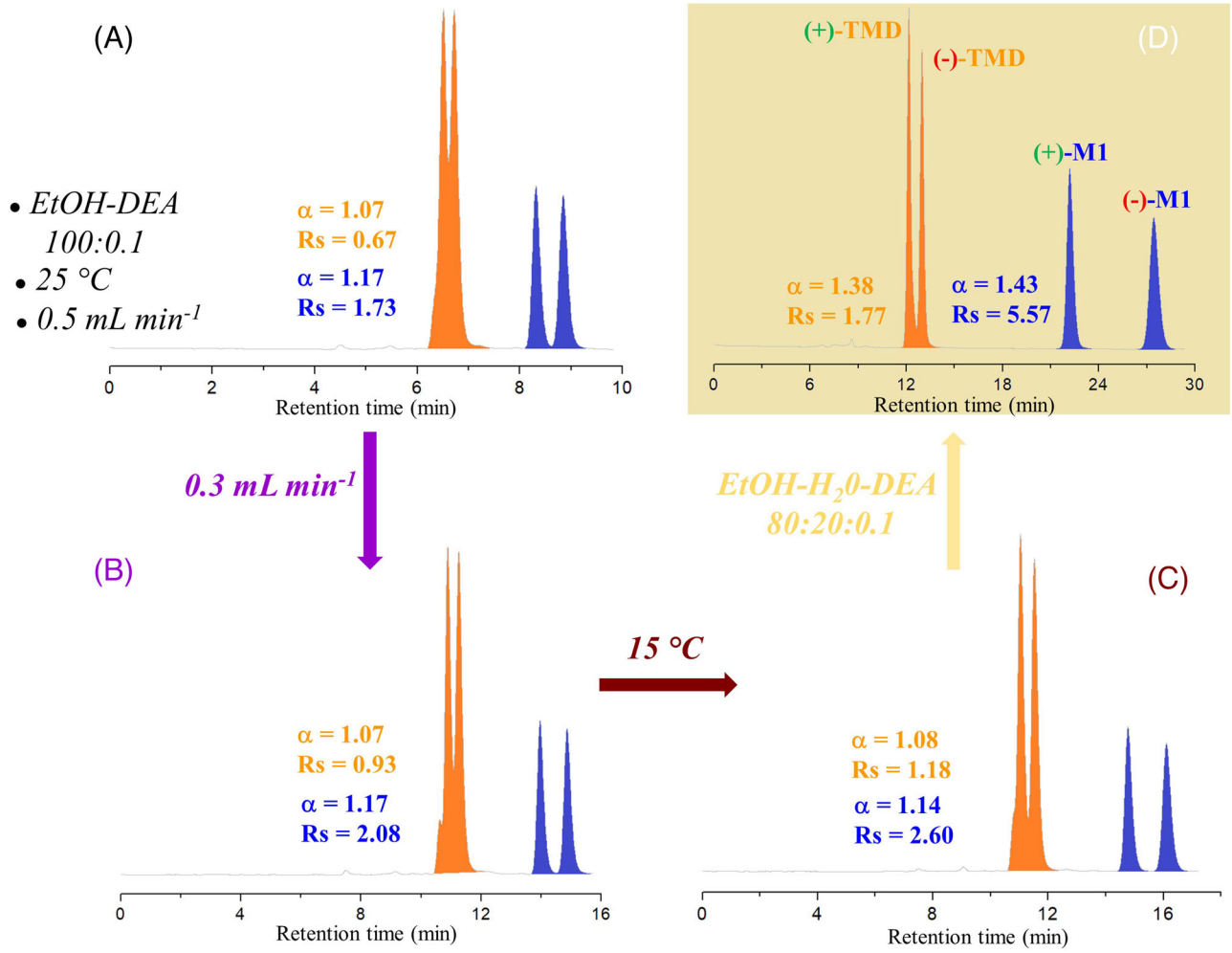

FIGURE 3 Chromatograms showing the high-performance liquid chromatography (HPLC) method development for the simultaneous separation of the enantiomers of tramadol and O-desmethyltramadol under reversed-phase mode. Chromatographic conditions: (A) eluent, ethanol-diethylamine (DEA) 100:0.1 (v/v); temperature, 25°C; flow-rate, 0.5 mL min^{-1}; (B) eluent, ethanol-DEA 100:0.1 (v/v); temperature, 25°C; flow-rate, 0.3 mL min^{-1}; (C): eluent, ethanol-DEA 100:0.1 (v/v); temperature, 15°C; flow-rate, 0.3 mL min^{-1}; d): eluent, ethanol-H_2O-DEA 80:20:0.1 (v/v/v); temperature, 15°C; flow-rate, 0.3 mL min^{-1}; (A–D): detection, UV at 270 nm

The proposed green reversed-phase HPLC method was highly linear in the concentration range 280–1.4 μg mL^{-1} for both racemic test compounds (correlation coefficient (r^2) values ≥ 0.999). The LOQ values were 1.14 and 1.16 μg mL^{-1} for (+)-TMD and (-)-TMD, and 1.33 and 1.40 μg mL^{-1} for (+)-M1 and (-)-M1, respectively.

4 | CONCLUSIONS

The present study demonstrated that the Chiralpak IG-3 column provides adequate chromatographic properties for the simultaneous separation of the enantiomers of TMD and M1 under normal- and reversed-phase conditions. Furthermore, the ACMPC-based CSP allows a baseline separation of the test compounds under isocratic elution using ethanol as an organic modifier. The use of the optimized chemo- and enantio-selective protocol may be a valid tool for an evaluation of the pharmacological effects of the enantiomers of TMD and M1 as well as their metabolism and the influence of genetic polymorphisms on their activity.

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CONFLICT OF INTEREST

The authors have declared no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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