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Research Article

Enhancing the Antibacterial Activity of Ciprofloxacin Against *Klebsiella pneumoniae* by Inhibiting the AcrAB-TolC Efflux Pump System Using Phenylalanine-arginine β-Naphthylamide

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Abstract

Background: *Klebsiella pneumoniae* shows varying degrees of resistance to antibiotic treatment; this resistance arises from multiple mechanisms, including the increased expression of multidrug (MDR) efflux pumps. An efflux pump inhibitor (EPI) is required to overcome this challenge and restore the effectiveness of antibiotics against the present MDR *K. pneumoniae*. ***Objective***: To investigate the synergistic effect of the EPI Phenylalanine-Arginine β-Naphthylamide (PaβN) and Ciprofloxacin (CIP) on the expression of efflux pump genes *AcrAB-TolC*, isolated from CIP-resistant *K. pneumoniae*. ***Methods***: 50 isolates of *K. pneumoniae* were collected from five different hospitals in Baghdad, Iraq. The minimum inhibitory concentration (MIC) values were determined for the CIP and CIP and PaßN combination using the broth micro-dilution method conducted on ten isolates resistant to CIP. Moreover, the expression level of *AcrA*, *AcrB*, and *TolC* genes from four selected isolates of *K. pneumoniae* were measured using quantitative real-time polymerase chain reaction (qRT-PCR). ***Results***: *K. pneumoniae* isolates showed that 40/50 (80%) were multidrug-resistant, and 54% of isolates were resistant to CIP. The MIC was reduced significantly from 2–8-fold in the isolates treated with PaβN and CIP compared to the CIP-treated group. The gene expression levels varied among the four selected isolates, with a slight decrease in *AcrAB-TolC* gene expression in some isolates treated with the CIP and PaβN combination compared to those treated with CIP alone. ***Conclusions***: The promising finding is the effectiveness of synergistic combinations between the antibiotic and efflux pump inhibitors in eliminating resistance of MDR bacteria.

Keywords: *AcrAB-TolC*, Antibiotic resistance, Efflux pump, Efflux pump inhibitor, *Klebsiella pneumoniae*, PaßN.

تعزيز نشاط المضاد البكتيري سيبروفلوكساسين ضد الكليبسلا الرئوية عن طريق تثبيط نظام مضخة التدفق *AcrAB-TolC* باستخدام فينيل ألانين-أرجينين-β نفثيلاميد

الخلاصة

الخلفية: تظهر الكليبسيلا الرئوية درجات متفاوتة من المقاومة للعلاج بالمضادات الحيوية. تنشأ هذه المقاومة من آليات متعددة، بما في ذلك التعبير المتزايد عن مضخات تدفق الأدوية المتعددة. مثبط مضخة التدفق (EPI) مطلوب للتغلب على هذا التحدي واستعادة فعالية المضادات الحيوية ضد *K. pneumoniae* المتعددة المقاومة للأدوية الحالية. **الهدف:** التحقق من التأثير التآزري بين مثبط مضخة التدفق فينيل ألانين أرجينين بيتا نفثيلاميد (PaβN) وسيبروفلوكساسين (CIP)، على التعبير الجيني لجينات مضخات التدفق (*AcrAB-TolC*) المعزولة من بكتيريا *K. pneumoniae* المقاومة لـ CIP. **الطرق:** تم جمع 50 عزلة من بكتيريا *K. pneumoniae* من خمسة مستشفيات مختلفة في بغداد، العراق. تم تحديد الحد الأدنى للتركيز المثبط (MIC) لـ CIP وتركيبة CIP and PaßN باستخدام طريقة التخفيف الجزئي التي أجريت على عشر عزلات مقاومة لـ CIP. وتم قياس مستوى التعبير عن جينات *AcrA* و*AcrB* و*TolC* لأربعة عزلات مختارة من *K. pneumoniae* باستخدام تفاعل البوليميريز المتسلسل الكمي في الوقت الحقيقي. **النتائج:** أظهرت عزلات *K. pneumoniae* أن 50/40 (80٪) كانت مقاومة للأدوية المتعددة، وأن 54٪ من العزلات كانت مقاومة لـ CIP. تم تخفيض MIC بشكل ملحوظ من 2 إلى 8 أضعاف في العزلات المعالجة بـ PaβN و CIP مقارنة بالمجموعة المعالجة بـ CIP. تباينت مستويات التعبير الجيني بين العزلات الأربع المختارة، مع انخفاض طفيف في التعبير الجيني *AcrAB-TolC* في بعض العزلات المعالجة بتركيبة CIP وPaβN مقارنة بتلك المعالجة بـ CIP وحده. **الاستنتاج:** هناك فعالية للتراكيب التآزرية بين المضادات الحيوية ومثبطات مضخة التدفق في القضاء على مقاومة البكتيريا للأدوية المتعددة.

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INTRODUCTION

Klebsiella pneumoniae is a highly pathogenic bacterium responsible for various serious infections, including pneumonia, urinary tract infections, wound infections, abscesses, sepsis, bloodstream infections, inflammation, and diarrhea [1]. The rise of multidrug-resistant (MDR) *K. pneumoniae* isolates has posed significant difficulties in managing and treating infections caused by this bacterium [2]. MDR efflux pumps are one of the most potent mechanisms that confer MDR of *K. pneumoniae*, particularly the efflux pumps that belong to the resistance-nodulation-cell division (RND) [3]. The tripartite RND *AcrAB-TolC* efflux pump is the most prevalent efflux in *K. pneumoniae*, consisting of the outer membrane

channel *TolC*, the inner membrane transporter *AcrB*, and the periplasmic membrane fusion protein *AcrA* [4]. An effective strategy to combat MDR efflux pumps involves using efflux pump inhibitors (EPIs). These molecules offer a promising approach to inhibit efflux pumps, thereby reducing antibiotic resistance [5]. EPIs work synergistically with antibiotics, allowing them to enter the cell more effectively and increasing the accumulation of the antibiotic within the cell [6]. The efflux pump inhibitor Phenylalanine-Arginine β-Naphthylamide (PaβN), also known as MC-207,110, is a low-molecular-weight dipeptide amide molecule [7] considered one of the most studied anti-efflux compounds in gram-negative bacteria. Mainly decrease the minimum inhibitory concentration (MIC) of quinolones, chloramphenicol, and tetracycline, which are substrates of *AcrAB* in MDR *K. pneumoniae* strains [6]. The mechanism of action of PaβN involves direct competitive binding, where PaβN competes with the substrates for the same binding site [8]. The level of efflux inhibition by PaβN depended on the substrate characteristics, especially the substrate with a binding site to efflux pumps [9]. The study aims to detect the efflux pump *AcrAB-TolC* of *K. pneumoniae* isolated from different clinical sources and to evaluate the synergetic effect of PaβN efflux pump inhibitor on the MIC levels of ciprofloxacin (CIP) antibiotic from the quinolone class and measure the gene expression of the *AcrAB-TolC* in the presence of CIP alone and in combination with PaβN at subinhibitory concentrations.

METHODS

Isolation and identification

A total of 50 *K. pneumoniae* isolates were collected from clinical specimens, such as urine, blood, sputum, burns, and wounds, from the microbiology laboratories of government and teaching hospitals in Baghdad, Iraq. All isolates were cultured on different mediums, including MacConkey agar and blood agar, and then incubated for 24 hours at 37°C. Colony morphology was used to identify isolates. All isolates were primarily identified based on the primary cultural characteristics of the colonies, including color, shape, texture, and size. Different methods are used to identify and classify bacterial isolates, such as culture on selective media (HiCrome *Klebsiella* Selective Agar Base). In addition, further identification was done using biochemical tests.

Antimicrobial susceptibility test

Disc diffusion assay was used to screen for antibiotic susceptibility in all 50 *K. pneumoniae* isolates according to Clinical and Laboratory Standards Institute (CLSI) guidelines 2023. Every isolate of *K. pneumoniae* was examined for susceptibility to fifteen distinct antibiotic drugs, including Amikacin (AK), Gentamicin (GEN), Amoxyclav® (Amoxycillin Clavulanic acid), Ampicillin (AMP), Azithromycin (AZM), Aztreonam (AZT), Cefotaxime, Ceftazidime (CAZ), Chloramphenicol (C), Ciprofloxacin (CIP), Levofloxacin (LE), Co-Trimoxazole® (sulfamethoxazole/trimethoprim) (COT), Imipenem (IMP), Nitrofurantoin (N), and Tetracycline (TE). The sizes of the inhibition zones were measured in millimeters in accordance with the CLSI 2023 to determine whether the inoculated plates were resistant, intermediate, or sensitive [10].

Minimum inhibitory concentration

Resistance of bacterial isolates to CIP and their MICs was measured using the broth microdilution method following the 2023 CLSI guidelines. The CIP concentrations were 512 µg/ml, 256 µg/ml, 128 µg/ml,64 µg/ml, 32 µg/ml, 16 µg/ml, 8.0 µg/ml, 4.0 µg/ml, 2.0 µg/ml, and 1.0 µg/ml. In accordance with Ohikhena *et al*. [11], the test was performed. Microtiter plates were incubated overnight at 37°C. After incubation, 20 μL of resazurin was added to each well and incubated for several minutes to observe any color change; when living cells reduce it, it changes to pink, and the minimum concentration that exhibited no noticeable growth is defined as the MIC.

Treatment with the efflux pump inhibitor

The synergistic effect of PaβN (MedChemExpress, USA) and CIP was tested as described under the Minimum Inhibitory Concentration section, but in the presence of 100 µg/ml PaβN. After adding CIP and bacterial cell inoculum, the microplate wells were treated with 2.0 µl of a 10 mg/ml PaβN stock solution. All the tested bacterial isolates were grown in Mueller Hinton broth with 100 µl/ml of PaβN to assess the acridity of the test and analyze the impact of PaβN on bacterial growth.

Molecular detection of AcrA, AcrB, and TolC

DNA was extracted from the *K. pneumoniae* isolates using the Presto™ Mini gDNA Bacteria Kit processed by Geneaid, Taiwan. The extracted DNA was detected by gel electrophoresis. In this study, conventional PCR was used for the detection of these efflux genes in *K. pneumoniae* isolates. Table 1 shows the primers used for the detection of efflux pump genes, and these primers were designed by the Primer 3 program. Table 2 demonstrates the PCR program where all 3 genes have the same annealing temperature of 58°C. PCR was performed in a total volume of 25 µl, and components are shown in Table 3.

RNA Extraction

Four bacterial isolates were selected based on their CIP resistance and were cultured in a Muller Hinton broth medium in the absence and presence of a sub-MIC concentration for the CIP group and the CIP + PaβN group. Total RNA was extracted from the non-treated, CIP-treated, and CIP + PaβN-treated group bacterial cells using a GENEzol™ TriRNA Pure Kit (Geneaid Biotech, Taiwan).

Table 1: The study used primers, along with their oligonucleotide sequences, amplicon sizes, and references.

Primer name		Sequence (5'-3')	Amplicon sizes (bp)	References
AcrA	F	TACACCAAAGTCACCTCGCC	148	Primer 3 program
	R	TCATTGCTCGACTGGGTGAC		
AcrB	F	TGTCCTCAAATGGCGACTCC	154	Primer 3 program
	R	AACGCTAATCCCTTGCTGCT		
Tol-C	F	CATCCTGAATCCCTGCCGTT	103	Primer 3 program
	R	CTCTAACGTCACCAGCGGTT		
Rpsl	F	TTTGGAGAACTGCGGACGAC	133	12
	R	CGCTCTGATGGAGTACGACG		

Table 2: Components of the conventional PCR reaction

PCR Master mix reaction components	Volume (µl)
Taq 2x Master mix	12.5
Forward primer (10 µM)	1.0
Reverse primer (10 µM)	1.0
DNA template	2.0
nuclease-free H2O	8.5
Total volume	25

Table 3: PCR amplification program for *AcrA, AcrB* and *Tol-C* efflux pump genes

Gene		Step	Temp (°C)	Time	Cycles
AcrA, AcrB, and TolC	1	Initial Denaturation	92	3 min	1
	2	Denaturation	92	30 sec	
	3	Annealing	58	30 sec	35
	4	Extension	72	1 min	
	5	Final Extension	72	3 min	1

Real-time quantitative reverse transcription PCR

For the measure expression of *AcrA*, *AcrB*, and *TolC* before and after treatment with CIP alone and CIP + PaβN, qRT-PCR was used. Four isolates that showed high resistance to CIP alone and reduced resistance to CIP in the presence of PaβN were selected to determine the expression of efflux genes. The primers used for expressing *AcrA*, *AcrB*, and *TolC* were the same as those used for conventional PCR. The housekeeping gene (*Rpsl*) primers used are listed in Table 1. Furthermore, the components of the quantitative qRT-PCR reaction are shown in Table 4. The quantification of AcrA, AcrB, and TolC gene expression was carried out using the ΔΔCT calculation, and the fold of gene expression was given as 2-ΔΔCT.

Table 4: Components of qRT-PCR

Component	Volume (µl)	Final Conc.
qPCR Master Mix (2X)	10	1X
RT Enzyme Mix (20X)	1	1X
Forward primer (10 pmol/µl)	0.8	0.4 µM
Reverse primer (10 pmol/µl)	0.8	0.4 µM
Template RNA	Variable	20 ng/µl
Nuclease-free water	Up to 20	

Statistical analysis

The Statistical Analysis System (SAS) program (2018) was used to detect the effect of different factors on study parameters. The chi-square test was used to compare the results. A *p*-value < 0.05 was considered statistically significant [13].

RESULTS

In total, 158 samples were collected from patients visiting five hospitals in Baghdad. All these samples were cultured on MacConkey agar, HiCrome Klebsiella Selective Agar Base, and blood agar plates for 24 hours at 37°C. Only 134 (90.5%) show positive bacterial species growth. All 50 *K. pneumoniae* isolates were tested for 15 antibiotics, and most of the isolates (80%, n=40) showed multidrug resistance. The study's findings indicate that all isolates exhibited total resistance (100%) to ampicillin and the highest resistance to cefotaxime (76%), ceftazidime (70%), and Co-Trimoxazole (sulfamethoxazole/trimethoprim) (72%). In comparison, the highest sensitivity rate against *K. pneumoniae* isolates was levofloxacin (16%) and chloramphenicol (10%), while the rate of resistance to CIP was 54%. and the correlation between *K. pneumonia's* antibiotic susceptibility and resistance was statistically significant (p<0.01). The minimum inhibitory concentrations (MICs) of CIP for ten multidrug-resistant isolates of *K. pneumoniae* showed variations in MICs among the isolates. The minimum inhibitory activity ranged from 1 to 512 µg/ml, as shown in Table 5.

Table 5: The MICs of CIP with and without PaβN in *K. pneumoniae* isolates

Isolates (n)	MICs of CIP (µg/ml)	MICs of CIP after adding PaβN (100 µg/ml) (µg/mL)	Fold reduction at MICs
1	2	1	2
2	512	128	4
3	4	1	4
4	128	16	8
5	2	<1	--
6	16	4	4
7	32	8	4
8	32	8	4
9	2	1	2
10	4	1	4

The treatment with 100 µg/ml of PaβN changes the susceptibility of isolates to CIP. The MICs of CIP dropped significantly by 2 to 8-fold, as shown in Table 5. 60% of isolates showed a 4-fold reduction, 20% showed a 2-fold reduction, and 10% showed an 8-fold reduction. In contrast, all the isolates that were cultured in the Mueller-Hinton broth, containing the PaβN (100 µg/ml) without any CIP, showed good growth of bacteria, indicating that the PaβN (100 µg/ml) did not have an antibacterial effect itself. The *AcrAB-TolC* genes were detected in all (100%) of the ten selected *K. pneumoniae* isolates by DNA amplification using the PCR technique and positively confirmed by agarose gel electrophoresis as shown in Figure 1. The expression of efflux pump genes for *AcrAB-TolC* in *K. pneumoniae* was also investigated in four isolates of *K. pneumoniae* in this study. The findings of the *AcrA*, *AcrB*, and *TolC* expressions are in Tables 6, 7, and 8. The results indicate significant variations in gene expression patterns across the isolates, as shown in Figure 2.

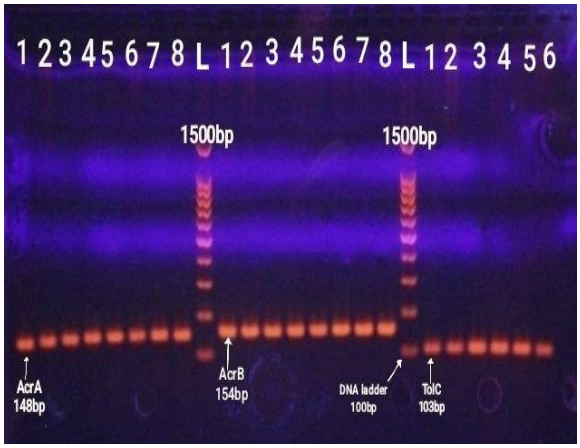

Figure 1: Agarose gel electrophoresis of conventional PCR amplification products of *AcrA, AcrB, and TolC* genes, in *K. pneumoniae* isolates. L: ladder (100bp); Lanes 1-8: PCR amplicons of *AcrA, AcrB, and TolC* (expected size 148bp, 154bp, and 103 bp, respectively, 2% agarose and 150V/30 min.

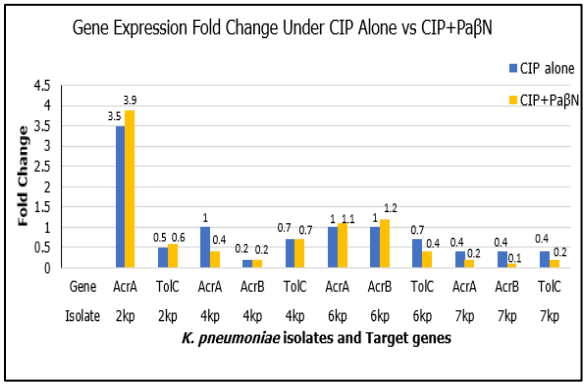

Figure 2: Gene expression fold change of *AcrA, AcrB,* and *TolC* in *K. pneumoniae* isolates treated with CIP alone and CIP+PaβN.

DISCUSSION

In this study, *the AcrAB-TolC* efflux pump was detected in all selected isolates, highlighting the widespread presence of this mechanism and its role in multidrug-resistant (MDR) resistance. The study's initial objective was to evaluate the synergistic effect between the efflux pump inhibitor PaβN and CIP in reducing the resistance of *K. pneumoniae* isolates to CIP. Phenotypically, through the broth microdilution method, the combination treatment with PaβN showed a significant reduction in the resistance of isolates to CIP.

Table 6: A- Regulation of *AcrA* expression

A- Before and after treatment with CIP								
Isolate code	Control			Antibiotic			ΔΔCt	Fold change
	hkg	*AcrA*	ΔCt	hkg	*AcrA*	ΔCt		
2Kp	13.12	17.21	4.09	11.2	13.48	2.28	-1.81	3.5
4Kp	16.73	18.23	1.5	11.9	13.41	1.51	0.01	1.0
6Kp	10.28	13.94	3.66	9.96	13.6	3.64	-0.02	1.0
7Kp	14.61	16.22	1.61	13.27	16.3	3.03	1.42	0.4
B- Before and after treatment with CIP combined with PaβN								
Isolate code	Control			Antibiotic			ΔΔCt	Fold change
	hkg	*AcrA*	ΔCt	hkg	*AcrA*	ΔCt		
2Kp	13.12	17.21	4.09	10.45	12.57	2.12	-1.97	3.9
4Kp	16.73	18.23	1.5	10.7	13.61	2.91	1.41	0.4
6Kp	10.28	13.94	3.66	9.49	13.06	3.57	-0.09	1.1
7Kp	14.61	16.22	1.61	9.83	13.64	3.81	2.2	0.2

Table 7: Regulation of *AcrB* expression

A- Before and after treatment with CIP								
Isolate code	Control			PaβN			ΔΔCt	Fold change
	hkg	*AcrB*	ΔCt	hkg	*AcrB*	ΔCt		
2Kp	13.12	16.35	3.23	11.2	14.35	3.15	-0.08	1.1
4Kp	16.73	17.61	0.88	10.7	14.31	3.61	2.73	0.2
6Kp	10.28	15.04	4.76	9.96	14.73	4.77	0.01	1.0
7Kp	14.61	16.33	1.72	13.27	16.29	3.02	1.3	0.4
B- Before and after treatment with CIP combined with PaβN								
Isolate code	Control			PaβN			ΔΔCt	Fold change
	hkg	*AcrB*	ΔCt	hkg	*AcrB*	ΔCt		
2Kp	13.12	16.35	3.23	10.45	14.45	4	0.77	0.6
4Kp	16.73	17.61	0.88	10.7	14.31	3.61	2.73	0.2
6Kp	10.28	15.04	4.76	9.49	14.01	4.52	-0.24	1.2
7Kp	14.61	16.33	1.72	9.83	14.3	4.47	2.75	0.1

Table 8: Regulation of *TolC* expression

A- Before and after treatment with CIP								
Isolate code	control			PaβN			ΔΔCt	Fold change
	hkg	*AcrB*	ΔCt	hkg	*AcrB*	ΔCt		
2Kp	13.12	23.09	9.97	11.2	22.22	11.02	1.05	0.5
4Kp	16.73	17.68	0.95	11.9	13.31	1.41	0.46	0.7
6Kp	10.28	12.63	2.35	9.96	12.73	2.77	0.42	0.7
7Kp	14.61	14.78	0.17	13.27	14.64	1.37	1.2	0.4
B- Before and after treatment with CIP combined with PaβN								
Isolate code	control			PaβN			ΔΔCt	Fold change
	hkg	*AcrB*	ΔCt	hkg	*AcrB*	ΔCt		
2Kp	13.12	23.09	9.97	10.45	21.05	10.6	0.63	0.6
4Kp	16.73	17.68	0.95	10.7	12.1	1.4	0.45	0.7
6Kp	10.28	12.63	2.35	9.49	13	3.51	1.16	0.4
7Kp	14.61	14.78	0.17	9.83	12.59	2.76	2.59	0.2

This aligns with previous studies, such as a study by Tian *et al*. [14] reported that most isolates showed a significant reduction from 2-fold to 4-fold in the presence of a PaβN and CIP, and another study by Vera-Leiva *et al*. [15] reported that 16 isolates of CIP-resistant isolates showed a 32-fold reduction in MIC. Genotypically, by determining their effect on the expression levels of efflux genes (AcrAB-TolC). The findings of this study showed varied expression profiles of efflux genes, upregulation, downregulation, and no change in expression with combination treatment. The differential response in (2Kp, 4Kp, and 7Kp) could imply that PaβN selectively affects certain components of the AcrAB-TolC, leading to a reduction in overall efflux activity, and also suggests that bacteria might upregulate certain components of the efflux pump as a response to stress [16] or when efflux pumps are inhibited, toxic cellular metabolites accumulate, leading to the upregulation or downregulation of specific efflux pump genes to restore hemostasis [17]. The decrease in 6Kp levels noted in both treatments indicate a limited reliance on efflux pumps and suggests the possibility of alternative resistance mechanisms. This study is one of the first studies to investigate and evaluate the expression of the *AcrAB-TolC* efflux pump in *K. pneumoniae* when treated with PaβN and ciprofloxacin. In contrast, other studies about other bacterial species reported an increase in the gene expression of *AcrAB-TolC* in *E. coli* after exposure to PaβN [18], while another study [19] showed a significant decrease in efflux genes of *P. aeruginosa* after exposure to PaβN. It investigated that the PaβN showed different expression profiles according to the nature of the competitive substrate (antibiotic) or gene mechanism, especially the PaβN has a dual effect on the outer membrane [20] and biofilm formation [6].

Conclusion

The effectiveness of synergistic combinations in eliminating MDR bacteria, as demonstrated in this study by the significant reduction of ciprofloxacin MIC values, presents an intriguing strategy for managing antimicrobial resistance (AMR). At the genetic level, the result shows that the PaβN significantly reduced the expression of efflux pump genes but indicates that the effectiveness of PaβN as an efflux pump inhibitor could differ depending on the expression levels of the efflux components. Due to the PaβN's multi-effects in combating MDR bacteria, there is a necessity for further investigation; It is recommended to test the PaβN alongside other different classes of antibiotics to target the expression of various efflux pump systems and virulence genes (such as membrane proteins, biofilm genes, etc.) or generate mutants or perform gene deletions to target the components of the efflux pump system to precisely understand the efflux mechanism.

Conflict of interests

No conflict of interest was declared by the authors.

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Data sharing statement

Supplementary data can be shared with the corresponding author upon reasonable request.

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