

Elucidation of the K32 Capsular Polysaccharide Structure and Characterization of the KL32 Gene Cluster of *Acinetobacter baumannii* LUH5549

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Abstract—Capsular polysaccharide (CPS), isolated from *Acinetobacter baumannii* LUH5549 carrying the KL32 capsule biosynthesis gene cluster, was studied by sugar analysis, Smith degradation, and one- and two-dimensional ¹H and ¹³C NMR spectroscopy. The K32 CPS was found to be composed of branched pentasaccharide repeats (K units) containing two residues of β-D-GalpNAc and one residue of β-D-GlcA (β-D-glucuronic acid) in the main chain and one residue each of β-D-Glcp and α-D-GlcpNAc in the disaccharide side chain. Consistent with the established CPS structure, the KL32 gene cluster includes genes for a UDP-glucose 6-dehydrogenase (Ugd3) responsible for D-GlcA synthesis and four glycosyltransferases that were assigned to specific linkages. Genes encoding an acetyltransferase and an unknown protein product were not involved in CPS biosynthesis. Whilst the KL32 gene cluster has previously been found in the global clone 2 (GC2) lineage, LUH5549 belongs to the sequence type ST354, thus demonstrating horizontal gene transfer between these lineages.

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Capsular polysaccharide (CPS) is produced on the cell surface of many bacterial pathogens including the nosocomial species, *Acinetobacter baumannii*. It serves as a major pathogenicity determinant by providing a layer protecting against host immune factors [1, 2]. It also protects cells from desiccation and other environmental stressors [3, 4]. CPS is composed of a long chain of oligosaccharide repeats (K units), which consist of two to six sugar residues of various types joined by specific gly-

cosidic linkages. The biosynthesis and export of CPS in *A. baumannii* is mostly determined by the genes at the chromosomal K locus (KL), which comes in diverse forms [5].

The K locus includes an operon of genes (*wza-wzb-wzc*) responsible for the CPS export, and a divergently transcribed region for the synthesis of specific K units. The latter includes genes for sugar synthesis, initiation of K-unit synthesis (*itr*), glycosyl transfer (*gtr*), K-unit translocation (*wzx*) and polymerization (*wzy*), and occasionally for addition of acetyl or pyruvyl groups (*atr/ptr*).

The arrangement of KL gene clusters carried by isolates from the Traub collection that were used to establish the original serotyping scheme for *A. baumannii* [6] had been examined previously [7]. However, these gene clus-

Abbreviations: CPS, capsular polysaccharide; KL, chromosomal K locus; K unit, oligosaccharide repeat; MPS, modified polysaccharide; PSgc, polysaccharide gene cluster.

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ters were originally described as polysaccharide gene clusters (PSgc) and annotated using a traditional nomenclature system. The structures of CPSs produced by many of these isolates have now been established and, for all of them, their sugar content and configuration correlate perfectly with the genes in the gene cluster found at the K locus [8-12]. Accordingly, the annotations of these gene clusters have been revised [8-14] in accordance with the more transparent and widely adopted K locus nomenclature system for *A. baumannii* CPS biosynthesis gene clusters [5].

In this work, we establish for the first time the CPS structure of another strain from this collection, LUH5549, which carries the KL32 gene cluster, previously designated PSgc21.

MATERIALS AND METHODS

Bacterial strain and cultivation of bacteria.

Acinetobacter baumannii LUH5549 was originally from the W. H. Traub collection at the Institut für Medizinische Mikrobiologie und Hygiene, Universität des Saarlandes (Saarland, Germany) and was kindly provided by Prof. Peter Reeves. The bacteria were cultivated in 2TY media overnight; the cells were harvested by centrifugation (10,000g, 20 min), washed with phosphate buffered saline, resuspended in aqueous 70% acetone, precipitated, and dried.

Isolation of CPS. CPS preparation was obtained by phenol–water extraction [15] of bacterial cells (1.1 g); the extract was dialyzed without layer separation and clarified from insoluble contaminants by centrifugation. The resulting solution was treated with cold (4°C) 50% aqueous CCl_3COOH ; after centrifugation, the supernatant was dialyzed against distilled water and freeze-dried to yield a crude CPS sample (370 mg). For CPS purification, this sample (120 mg) was heated with 2% aqueous AcOH at 100°C for 2 h, and CPS (27 mg) was purified by gel-permeation chromatography on a Sephadex G50 Superfine column 60 × 2.5 cm in 0.1% aqueous AcOH; eluted fractions were monitored with a differential refractometer (Knauer, Germany).

Monosaccharide analysis. Alditol acetates [16] were obtained by CPS hydrolysis with 2 M CF_3COOH (120°C, 2 h) and analyzed by gas-liquid chromatography on an HP-5 capillary column with a Maestro (Agilent 7820) chromatograph (Interlab, Russia) using a temperature gradient of 160°C (1 min) to 290°C at 7°C/min. Glucuronic acid was identified by anion-exchange chromatography on a DA×8 resin column (7 × 0.4 cm) in 5 mM potassium phosphate buffer (pH 3) at 70°C; elution was monitored using bichinchoninic acid assay.

Smith degradation. CPS sample (12 mg) was oxidized with NaIO_4 (29 mg in 1.5 ml water) at 20°C for 72 h in the dark and then reduced with NaBH_4 (30 mg) for

24 h; water was evaporated and boric acid was removed by evaporation with 10% aqueous AcOH in methanol (three times). The degradation product was desalted on a Sephadex G-25 column (108 × 1.2 cm) in water; elution was monitored as described above. The obtained polymer was hydrolyzed with 2% aqueous AcOH (100°C, 2 h), and the products of hydrolysis were fractionated by gel-permeation chromatography on Sephadex G-25 as described above to yield modified polysaccharide (MPS) (2.6 mg).

NMR spectroscopy. The samples were deuterium-exchanged by freeze-drying from 99.9% D_2O and then examined after dissolving in 99.95% D_2O . The NMR spectra were recorded on an Avance II 600 MHz spectrometer (Bruker, Germany) at 60°C. Sodium 3-trimethylsilylpropanoate-2,2,3,3- d_4 (δ_{H} 0, δ_{C} -1.6) was used as an internal reference for calibration. 2D NMR spectra were obtained using standard Bruker software; NMR data were acquired and processed with the Bruker TopSpin 2.1 program. A spin-lock time of 60 ms and a mixing time of 150 ms were used in TOCSY and ROESY experiments, respectively. A 60-ms delay was used for the evolution of long-range coupling to optimize ^1H , ^{13}C HMBC experiments.

Bioinformatics analysis. Short reads for the LUH5549 genome sequence were obtained from the Sequence Read Archive (SRA number DRS005644) and assembled into contigs using SPAdes [17]. The KL32 gene cluster was identified between the *fkpA* and *lldP* genes and re-annotated using the nomenclature system described previously [5]. CAZy (<http://www.cazy.org/>) [18] and Pfam (<https://pfam.xfam.org/>) [19] databases were used to assign encoded proteins to their respective biosynthesis roles. The assembled sequence and the newly assigned annotations are available in GenBank under accession number KC526897.2. The genome sequence was assigned to a sequence type (ST) using the Pasteur MLST scheme for *A. baumannii* (https://pubmlst.org/bigsubdb?db=pubmlst_abaumannii_pasteur_seqdef).

RESULTS AND DISCUSSION

The sequence of the PSgc21 CPS biosynthesis gene cluster from *A. baumannii* LUH5549 (GenBank accession number KC526897.1) was found to be incomplete as it lacked the *wza-wzb-wzc* export genes, indicating a potential assembly issue. As the sequence had been previously assembled from short reads using Velvet 2.0 [7], these short reads (SRA number DRS005644) were reassembled into contigs using SPAdes. The complete sequence of the gene cluster between the conserved *fkpA* and *lldP* genes that flank the K locus was identified and found to be 99.56% identical to the KL32 gene cluster previously described for the *A. baumannii* Vietnamese isolate, BAL_058 (GenBank accession number

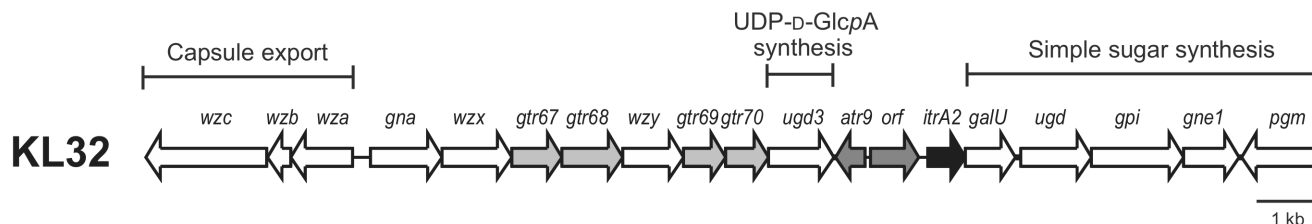


Fig. 1. The KL32 capsule biosynthesis gene cluster from *A. baumannii* LUH5549. Arrows indicate the direction of transcription and are drawn to scale using the sequence from GenBank accession number KC526879.2. Genes for glycosyltransferases (*gtr*) are depicted in light grey; initiating transferase gene (*itrA2*) is shown in black. Genes shown in dark grey (*atr9*, *orf*) have no established role in the synthesis of the K32 CPS.

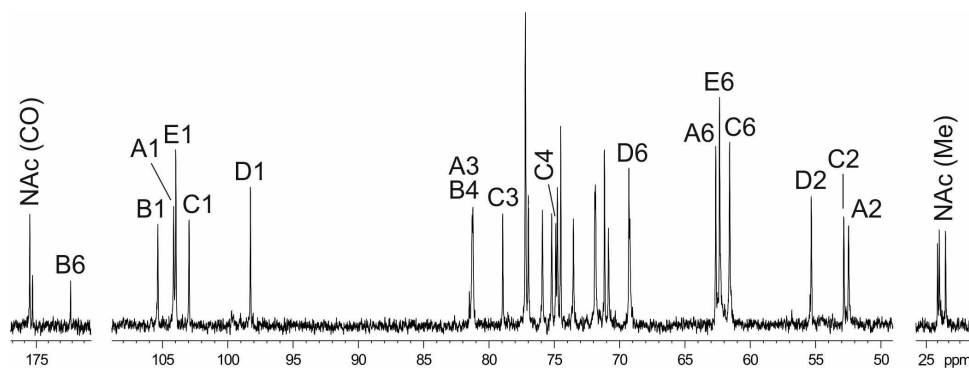


Fig. 2. ^{13}C NMR spectrum of the K32 CPS from *A. baumannii* LUH5549. Numbers refer to carbons in sugar residues denoted by letters as indicated in the table and Fig. 3.

KT359615.1) [20]. Hence, the LUH5549 gene cluster was renamed KL32 and its genes were reannotated according to the established nomenclature system [5]. The updated sequence and the annotations were deposited in the GenBank under accession number KC526897.2.

KL32 (Fig. 1) includes genes responsible for the K unit processing (*wzy* and *wzx*) and a *galU-ugd-gpi-gne1-pgm* gene module responsible for the synthesis of simple sugars, such as UDP-D-Glcp, UDP-D-GlcpNAc, and UDP-D-GalpNAc. Adjacent to *galU*, there is an *itrA2* gene known to be responsible for initiating K unit synthesis by adding a D-GalpNAc residue as the first sugar of the K unit to the lipid carrier in the inner membrane [21]. The central region of KL32 also includes four predicted glycosyltransferase genes (*gtr67*, *gtr68*, *gtr69*, *gtr70*) for four internal K unit sugar linkages, an acetyltransferase gene (*atr9*), and a gene encoding an unidentified protein product (Orf).

In the central region, another *ugd* gene, designated *ugd3*, is also present. Ugd3 (GenPept accession number AHB32286.1) is only 20% identical (74% coverage) to the product of the *ugd* gene (GenPept accession number AHB32291.1) located in the simple sugar synthesis gene module, which was previously predicted to be responsible for the conversion of UDP-D-Glcp to UDP-D-glucuronic acid (D-GlcpA) [5]. However, though *ugd* is a

feature common to all *A. baumannii* KL gene clusters, its role in the CPS synthesis has never been established. Ugd3 is also 27% identical (71% coverage) to the second Ugd type (Ugd2) identified in the species, encoded in the central region of the KL20 and KL21 gene clusters. K20 and K21 CPSs contain D-GlcpA, which correlates with the presence of Ugd2 (GenPept accession numbers AUG44319.1 and AIT56461.1) [22]. The presence of *ugd3* also in the central region of KL32 specific to the CPS synthesis suggests that the K32 CPS may also contain D-GlcpA.

For the structure elucidation, CPS was isolated from the bacterial mass by phenol/water extraction followed by heating with 2% AcOH to remove contaminating short-chain lipopolysaccharides and then purified by gel-permeation chromatography on Sephadex G-50 Superfine. Sugar analysis using gas-liquid chromatography of alditol acetates derived after full acidic hydrolysis of CPS revealed the presence of Glc, GlcNAc, and GalNAc in the 1 : 1.6 : 1.8 ratio (detector response). In addition, GlcA was identified by anion-exchange chromatography. The absolute configuration of the monosaccharides was not determined chemically but inferred from the genetic data (see above).

The ^1H NMR and ^{13}C NMR (Fig. 2) spectra of the CPS showed signals for five monosaccharide residues and

^1H and ^{13}C NMR chemical shifts (ppm) of the K32 CPS from *A. baumannii* LUH5549

Sugar residue		C-1 <i>H-1</i>	C-2 <i>H-2</i>	C-3 <i>H-3</i>	C-4 <i>H-4</i>	C-5 <i>H-5</i>	C-6 <i>H-6</i>
CPS							
$\rightarrow 3$)- β -D-GalpNAc-(1 \rightarrow	A	104.1 <i>4.54</i>	52.5 <i>3.83</i>	81.3 <i>3.89</i>	69.2 <i>4.06</i>	75.9 <i>3.59</i>	62.6 <i>3.74; 3.82</i>
$\rightarrow 4$)- β -D-GlcpA-(1 \rightarrow	B	105.3 <i>4.58</i>	73.5 <i>3.40</i>	75.2 <i>3.64</i>	81.2 <i>3.84</i>	74.8 <i>4.01</i>	172.1
$\rightarrow 3,4$)- β -D-GalpNAc-(1 \rightarrow	C	102.9 <i>4.53</i>	52.8 <i>4.06</i>	79.0 <i>3.84</i>	74.9 <i>4.22</i>	77.0 <i>3.73</i>	61.6 <i>3.64; 3.68</i>
$\rightarrow 6$)- α -D-GlcpNAc-(1 \rightarrow	D	98.3 <i>4.86</i>	55.3 <i>3.90</i>	71.9 <i>3.87</i>	70.9 <i>3.71</i>	72.0 <i>4.34</i>	69.3 <i>4.06; 4.32</i>
β -D-Glcp-(1 \rightarrow	E	104.0 <i>4.47</i>	74.5 <i>3.35</i>	77.2 <i>3.49</i>	71.2 <i>3.40</i>	77.2 <i>3.43</i>	62.4 <i>3.72; 3.91</i>
MPS							
$\rightarrow 3$)- β -D-GalpNAc-(1 \rightarrow	A	103.7 <i>4.58</i>	52.4 <i>3.97</i>	81.1 <i>3.85</i>	69.1 <i>4.12</i>	76.0 <i>3.64</i>	62.3 <i>3.76; 3.78</i>
$\rightarrow 4$)- β -D-GlcpA-(1 \rightarrow	B	105.4 <i>4.50</i>	73.9 <i>3.36</i>	75.1 <i>3.57</i>	81.1 <i>3.76</i>	77.9 <i>3.68</i>	
$\rightarrow 3$)- β -D-GalpNAc-(1 \rightarrow	C	102.3 <i>4.51</i>	52.4 <i>3.95</i>	80.7 <i>3.80</i>	69.3 <i>4.16</i>	76.0 <i>3.70</i>	62.3 <i>3.77; 3.81</i>

Notes: ^1H NMR chemical shifts are italicized. Chemical shifts for the *N*-acetyl groups are δ_{H} 1.96-2.06, δ_{C} 23.3-24.0 (Me) and 175.3-175.5 (CO).

three *N*-acetyl groups. Assignment of the spectra using two-dimensional ^1H , ^1H COSY, ^1H , ^1H TOCSY, ^1H , ^1H ROESY, ^1H , ^{13}C HSQC, and ^1H , ^{13}C HMBC experiments revealed spin systems for one residue each of β -Glc (unit **E**), β -GlcA (unit **B**), α -GlcNAc (unit **D**), and two residues of β -GalNAc (units **A** and **C**), all being in the pyranose form (table). The α -*gluco* configuration of unit **D**, β -*gluco* configuration of units **B** and **E**, and β -*galacto* configuration of units **A** and **C** were inferred from the $^3J_{\text{H,H}}$ coupling constants for the ring protons, which were estimated from the one- and two-dimensional NMR

spectra. The anomeric configuration of units **A-C** and **E** was confirmed by H-1/H-5 correlations in the ROESY spectrum.

Low-field positions of signals for C-3 of units **C** and **A** at δ 79.0 and 81.3, C-4 of units **C** and **B** at δ 74.9 and 81.2, respectively, as well as C-6 of unit **D** at δ 69.3 (table), as compared with their positions in the corresponding non-substituted monosaccharides [23, 24], showed that the CPS is branched and allowed determination of the glycosylation pattern in the K unit. Accordingly, the ^{13}C NMR chemical shifts for

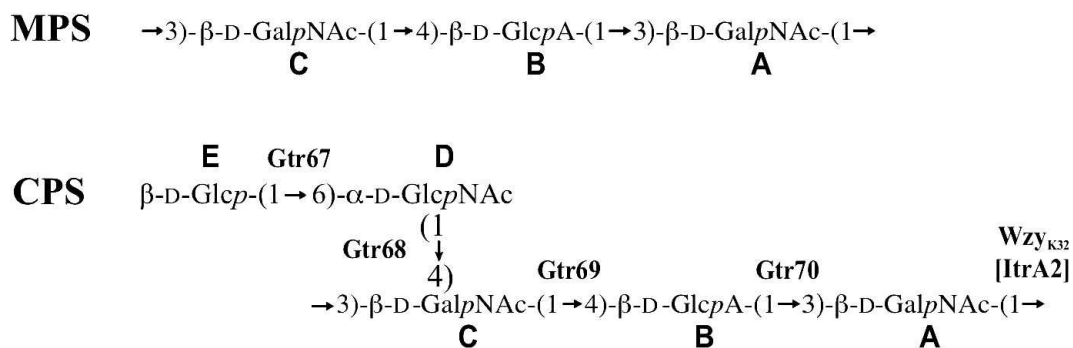


Fig. 3. Structures of the K32 CPS from *A. baumannii* LUH5549 and MPS derived by Smith degradation of the CPS. Glycosyltransferases, Wzy polymerase, and ItrA2 initiating transferase are shown near the linkage they presumably form.

C-2,3,4,5,6 of the terminal unit **E** (table) were close to those of unsubstituted β -glucopyranose [23].

The ^1H , ^{13}C HMBC spectrum of the CPS [Fig. S1; see Supplement to this paper on the web sites of the journal (<http://protein.bio.msu.ru>) and Springer (Link.springer.com)] showed correlations between the anomeric protons and linkage carbons at δ 4.47/69.3, 4.53/81.2, 4.54/79.0, 4.58/81.3, and 4.86/74.9, which were assigned to the **E** H-1/**D** C-6, **C** H-1/**B** C-4, **A** H-1/**C** C-3, **B** H-1/**A** C-3, and **D** H-1/**C** C-4 correlations, respectively. The glycosylation pattern of the CPS was confirmed by correlations of the anomeric protons and anomeric carbons with protons at the linkage carbons in the ^1H , ^1H ROESY (see Fig. S2 in the Supplement) and ^1H , ^{13}C HMBC spectra, respectively.

Therefore, the K32 CPS of *A. baumannii* LUH5549 has the structure shown in Fig. 3, which, to our knowledge, is unique among the known bacterial polysaccharide structures deposited in the Bacterial Carbohydrate Structure Database (BCSD; <http://csdb.glycoscience.ru/bacterial/>) [25]. This structure was confirmed by Smith degradation, which yielded a modified polysaccharide (MPS). Its structure (Fig. 3) was established by one- and two-dimensional NMR spectroscopy as described above for the CPS (see the table for assigned ^1H and ^{13}C NMR chemical shifts of the MPS).

The K32 CPS was found to be composed of pentasaccharide K units that include the common sugars, D-GalpNAc, D-GlcpNAc, and D-Glcp, as well as D-GlcpA, as predicted. We previously suggested that the *ugd* gene present in the module for the simple sugar synthesis may be redundant with respect to the CPS production [5], and that D-GlcpA can only be a component of the *A. baumannii* CPS when a second *ugd* gene is present in the corresponding KL gene cluster [22]. Indeed, KL32 contains a *ugd3* gene in the central region, and as a result, K32 CPS contains D-GlcpA.

As the CPS structure determined includes two D-GalpNAc residues (Fig. 3), it was unclear which residue was the first monosaccharide of the K32 unit that is added by ItrA2 (GenPept accession number AHB32289.2). However, the identity of the first sugar was determined by examining the linkage formed by the Wzy_{K32} polymerase responsible for joining K units together into the CPS chain, as this linkage would connect the first and the last sugars in the chain of K units. Wzy_{K32} (GenPept accession number AHB32283.1) was found to be 28% identical to Wzy_{K116} encoded by *A. baumannii* KL116 (GenBank accession number MK399425.1), which forms the β -D-GalpNAc-(1 \rightarrow 3)-D-GalpNAc linkage [26]. As an identical linkage is present in the K32 unit, Wzy_{K32} was assigned to it. Therefore, the structure of the K32 unit begins with the monosubstituted D-GalpNAc residue as drawn in Fig. 3.

The assembly of this K unit would therefore require three inverting glycosyltransferases to form the three β

linkages, and one glycosyltransferase with a retaining mechanism for the only α linkage. Only one glycosyltransferase encoded by KL32, Gtr68_{K32} (GenPept accession number AHB32282.2), was identified as a retaining enzyme belonging to the GT4 family of retaining glycosyltransferases in the CAZy database [18]. Therefore, Gtr68_{K32} was assigned to the α -D-GlcpNAc-(1 \rightarrow 4)-D-GalpNAc linkage.

The remaining glycosyltransferases were assigned to linkages in the K32 unit based on their homology to enzymes with known or predicted activities as follows. Gtr70_{K32} (GenPept accession number AHB32285.1) is 54% identical to WdbN from the *Escherichia coli* O143 O-antigen gene cluster (GenPept accession number STM86374.1), and the O143 structure contains the β -D-GlcpA-(1 \rightarrow 3)-D-GlcpNAc linkage [27]. A similar linkage, β -D-GlcpA-(1 \rightarrow 3)-D-GalpNAc, is present in K32 (Fig. 3), and therefore Gtr70_{K32} was assigned to this linkage. Gtr67_{K32} (GenPept accession number AHB32281.1) was found to be 27% identical to Gtr75_{K37} encoded by *A. baumannii* KL37 gene cluster (GenBank accession number KX712115.1). The structure of K37 is known [26], and Gtr75_{K37} was predicted to catalyze formation of the β -D-Glcp-(1 \rightarrow 6)-D-GalpNAc linkage. Hence, the β -D-Glcp-(1 \rightarrow 6)-D-GlcpNAc side branch of K32 would be formed by Gtr67_{K32}. Finally, Gtr69_{K32} (GenPept accession number AHB32284.1) that falls into the glycosyl_transf_2 family (Pfam PF00535) of glycosyltransferases, returns no significant hits to other proteins in BLASTp. However, as there was only one linkage left to be assigned in the K32 structure, Gtr69_{K32} was assigned to the β -D-GalpNAc-(1 \rightarrow 4)-D-GlcpA linkage (Fig. 3).

Since the K32 unit contains no acetyl or other acyl groups, and all its structural features were attributed to the presence of other genes encoded by KL32, a role for Atr9 (GenPept accession number AHB32287.2) and Orf (GenPept accession number AHB32288.2) in the synthesis of the CPS could not be established.

Previously, the KL32 gene cluster had been identified in the widely disseminated clonal group, Global Clone 2 (GC2; equivalent to sequence type ST2 in the Pasteur MLST scheme) [20]. We found that *A. baumannii* LUH5549 belongs to another type, ST354, suggesting that the KL32 gene cluster is distributed amongst multiple distinct clonal lineages.

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Conflict of interest. The authors declare no conflict of interest in financial nor in any other area.

Ethical approval. This article does not contain any studies with human participants or animals performed by any of the authors.

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