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Genomic analysis of extra-intestinal pathogenic *Escherichia coli* urosepsis

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Abstract

Urosepsis is a bacteraemia infection caused by an organism previously causing an infection in the urinary tract of a patient, a diagnosis which has been classically confirmed by culture of the same species of bacteria from both blood and urine samples. Given the new insights afforded by sequencing technologies into the complicated population structures of infectious agents affecting humans, we sought to investigate urosepsis by comparing the genome sequences of blood and urine isolates of *Escherichia coli* from five patients with urosepsis. The results confirm the classical urosepsis hypothesis in four of the five cases, but also show the complex nature of extra-intestinal *E. coli* infection in the fifth case, where three distinct strains caused two distinct infections. Additionally, we show there is little to no variation in the bacterial genome as it progressed from urine to blood, and also present a minimal set of virulence genes required for bacteraemia in *E. coli* based on gene association. These suggest that most *E. coli* have the genetic propensity to cause bacteraemia.

Keywords: *Escherichia coli*, extra-intestinal pathogenic, *E. coli*, genomics, urosepsis, virulence
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Introduction

Extra-intestinal pathogenic *Escherichia coli* (ExPEC) infections are now the most common cause of healthcare-associated infection, having overtaken cases of methicillin-resistant *Staphylococcus aureus* in recent years (http://www.hpa.org.uk/Topics/InfectiousDiseases/InfectionsAZ/ESBLs/), with a marked increase in the number of cases of bacteraemia caused by ExPEC being reported worldwide [1]. A large number of ExPEC bacteraemia infections are believed to be cases of urosepsis, where the bacteraemia stems from untreated urinary tract infections (UTI). In urosepsis, *E. coli* causing uncomplicated UTI in the bladder ascend to the kidneys and then enter the bloodstream via the fluid exchange system in the renal glomeruli [2] leading to bacteraemia, which can have mortality rates as high as 33% in elderly hospitalized patients [3].

Comprehensive studies of the pathogenesis of ExPEC have led to a detailed understanding of the molecular basis of this process at the microbial level. The current accepted paradigm for ascending UTI involves initial exposure to the urethra and travel to the bladder via flagella-mediated motility [4], whereby attachment to bladder epithelial cells is mediated by type 1 fimbriae and possibly a combination of several other accessory adhesins [5–7]. The bacteria then invade the epithelium and form intracellular bacterial communities, allowing subsequent detachment from the biofilm and escape [8]. At this stage the bacteria may further ascend the urinary tract to the kidneys where they cause pyelonephritis, with the production of P fimbriae by the bacteria considered essential for this progression [9]. From here the proximity to the blood–fluid exchange system of the renal glomeruli allows spillage of bacteria into the bloodstream and establishment of bacteraemia.

Despite the wealth of information from *in vitro* and animal challenge studies on the urosepsis model, all knowledge of urosepsis in humans is based on the positive culture of *E. coli* from urine and blood samples taken from the patient within a

close time frame, and the extrapolation of experimental data. Given the advances in sequencing technology we sought to provide a definitive examination of urosepsis in humans by genome sequencing ExPEC isolated from urine and blood samples of five patients with urosepsis. Our results confirm that the urosepsis hypothesis is applicable to humans but also highlights a case where the bacteraemia was caused by a strain different from two isolated strains co-infecting the urinary tract. Additionally our genomic data, supported by screening of a large bacteraemia isolate collection, questions the requirement for P fimbriae and uncovers a minimum virulence-associated gene set associated with sepsis that appears to be conserved across the species.

Methods

Five patients were identified as having urosepsis across two hospitals in the East Midlands region of the UK. The urosepsis definition was based on the isolation of a pure culture of *E. coli* from the blood sample of a patient, from whom a pure culture of *E. coli* had been isolated from a clinical urine sample within the previous 48 h. The genomes of the ten isolates were then sequenced using the Illumina Hiseq 2000 platform, assembled using SOAPdenovo and annotated using RATT. Single nucleotide polymorphisms (SNPs) were called by mapping using the SMALT program and SAMtools utilities. The SNPS were then manually filtered using Artemis to only call SNPs with a minimum depth of 8, minimum quality score of 30, and minimum allele frequency of 0.95. All remaining SNPs were then manually checked against BAM files in Artemis to ensure that they were reliable and not in areas at the ends of reads or in phage, insertion sequences or transposons, which are often difficult to accurately map against.

The multilocus sequence typing of the isolates was determined according to the Achtman typing scheme (http://mlst.ucc.ie/).

Results

Multilocus sequence typing data showed that in four of the five cases the same sequence type (ST) was isolated from urine and blood (Table 1), and that all four ST belonged to the phylogroup B2 high pathogenic lineage of ExPEC [10]. Patient 5 showed the presence of the pandemic multidrug-resistant *E. coli* ST131 [11] in the urine sample, but a classically non-pathogenic phylogroup A *E. coli* ST10 in the blood sample. To investigate this further a selection of colonies from the urine plate were subjected to multilocus sequence typing uncovering the presence of two different strains in the urine sample, namely ST131 and an ST127, with no detection of the ST10 strain (Table 1). The genome sequence data for all strains are represented diagrammatically in Fig. 1.

To definitively confirm that the blood and urine *E. coli* isolates from patients 1–4 were indeed the same, SNP typing was performed on the eight strains. In the absence of a reference strain suitably close to all four identified STs to allow common SNP typing we adopted the strategy of mapping the Illumina sequencing data of the blood isolate against a SOAPdenovo annotated genome [12] of the corresponding urine isolate. This was based on the rationale that the urine isolate is the primary infectious agent, which then progresses and possibly evolves during the ascension to bacteraemia. The resulting SNP profile for the four matched cases (Table 2) provides evidence that the strains causing the UTI are the same as those causing the bacteraemia infection, with isolates from patient 3 having no detectable difference across the genome using our stringency settings. Additionally we could not detect any sufficiently robust insertions or deletions across any of the four cases using our selection criteria. We determined the location of the seven SNPs across patients 1, 2 and 4 using the annotated urine isolate genomes. All but one of the seven SNPs identified in our three remaining cases were non-synonymous mutations and five of the SNPs, present across all three cases were situated in the region of the chromosome encoding the *yeeV*/*yeeU* toxin–antitoxin system, with mutations in *yeeV* as well as the surrounding genes *yeeT* and *yeeW*, which encode hypothetical proteins. This is suggestive of a selection effect on the *yee* locus during ascension from the bladder to bloodstream, as seen in other loci of ExPEC strains isolated from individual patients [13]. *In sillico* analysis of the SNP locations in the genes in P1, P2 and P4 (Fig. 2) failed to uncover any biological significance because of the lack of characterization of the encoded proteins, including absence of defined Pfam domains. In a further attempt to characterize the biological relevance of the SNPs we compared virulence phenotypes of our matched strains, assaying *in vitro* cell adhesion and invasion, biofilm formation, motility, serum resistance and cell morphology (Table 3). The only notable differences were in serum resistance, particularly in patient 1, which curiously also contains most SNPs between the blood and urine isolate.

We performed comparative genomics across our five bacteraemia isolate genome sequences paying attention to genes previously described as ExPEC virulence factors in published literature. Most strikingly isolates from patient 4 (ST1917 complex) and patient 5 (ST10) did not carry the *pap* genes encoding P fimbriae, which has been previously considered essential for ascending UTI [9]. Similarly the high

TABLE 1. Details of patients and isolates which formed the study

Patient number and source of isolate	Patient information	*Escherichia coli* sequence type	Extended spectrum β-lactamase status by PCR
Patient 1 urine (P1U)	92-yearold female catheterized patient	ST14 complex (ST550)	–
Patient 1 blood (P1B)		ST14 complex (ST550)	–
Patient 2 urine (P2U)	92-year-old female catheterized patient	ST131	–
Patient 2 blood (P2B)		ST131	–
Patient 3 urine (P3U)	81-year-old female patient	ST73 complex (ST1262)	–
Patient 3 blood (P3B)		ST73 complex (ST1262)	–
Patient 4 urine (P4U)	51-year-old female patient. No	ST1917	–
Patient 4 blood (P4B)	underlying co-morbidities	ST1917	–
Patient 5 urine (P5U1)	75-year-old male catheterized patient	ST131	+
Patient 5 urine (P5U2)		ST127	–
Patient 5 blood (P5B)		ST10	–

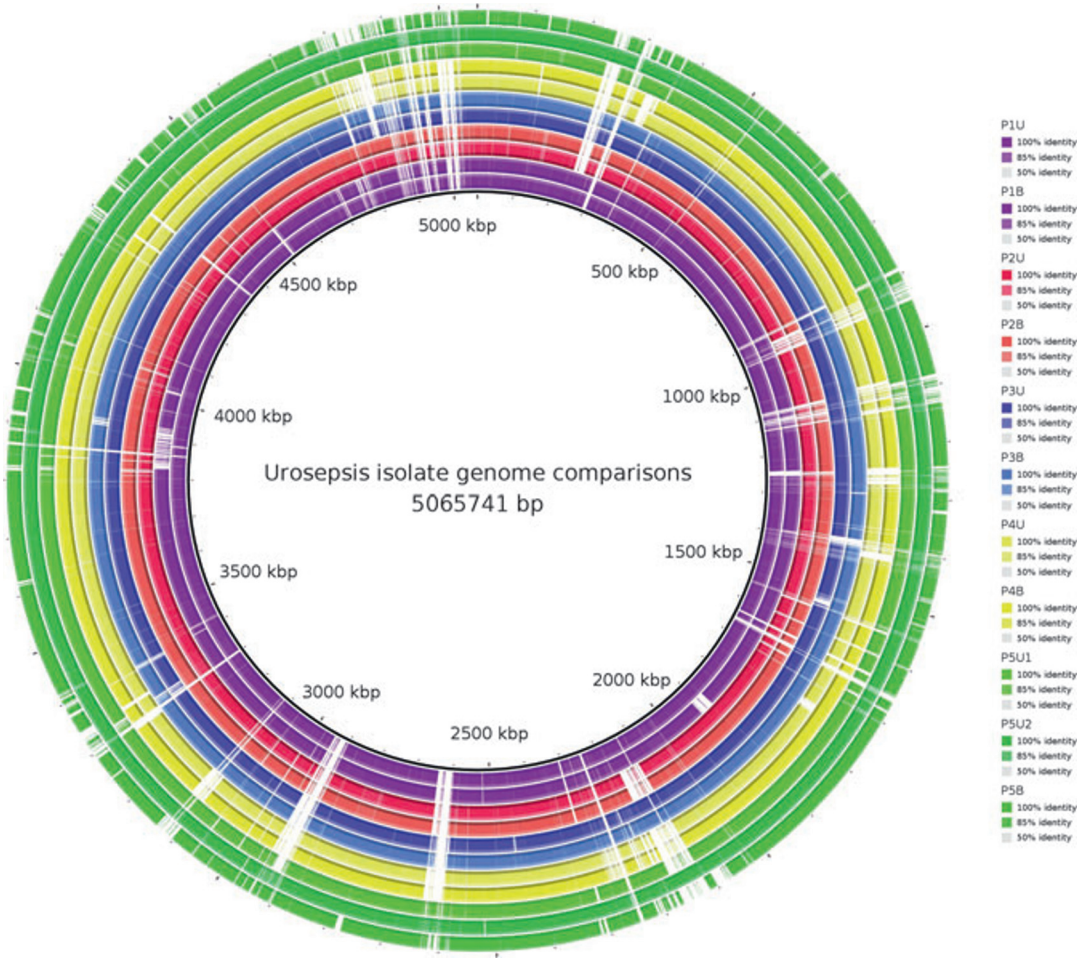

FIG. 1. Comparative genome representation of the 11 genomes sequenced from five patients in our study. The isolates are colour coded by patient, and show the clear relationship between the blood and urine isolates in patients 1–4, and the presence of three distinct strains in patient 5.

pathogenicity island HPI is also missing from ST10, as would be expected for a phylogroup A strain, precluding it from being considered as a necessary element for bacteraemia but not from ascending UTI and bacteraemia given we cannot prove the previous presence of the ST10 in the patient's urinary tract. Analysis of genes present across all five isolates identified type 1 fimbriae (*fim*), flagella, the *fes/fep* iron acquisition system, the *cus* heavy metal efflux system, and curli fimbriae (*csg*) as being a minimum virulence-associated gene requirement for an ExPEC strain to be able to cause bacteraemia. Screening with PCR for all five of the common bacteraemia loci across a collection of 150 bacteraemia and 150 UTI ExPEC isolates (collected over a 3-month period in 2011 in Nottingham University Hospital from unrelated clinical episodes)

TABLE 2. Single nucleotide polymorphisms (SNPs) identified within blood isolates when compared with their corresponding urine isolates

Patient isolates	SNP position	Gene	SNP[a]	Substitution
1	1453958	Hypothetical protein – Vgr-like family	G→T	Ser→Ile
	1786733	Putative transport protein *yebQ*	G→T	Gly→Val
	4531403	Toxin of toxin/anti-toxin system *yeeV*	C→T	Gly→Ser
	4531448	Toxin of toxin/anti-toxin system *yeeV*	A→G	Ser→Pro
2	4163357	Hypothetical protein in toxin/anti-toxin operon *yeeW*	G→T	Synonymous
3	No detectable SNPs	N/A		
4	1039343	Hypothetical protein in toxin/anti-toxin operon *yeeT*	G→A	Gly→Glu
	1039391	Hypothetical protein in toxin/anti-toxin operon *yeeT*	G→T	Gly→Val

[a]SNP differences are called with respect to the urine strain genome Fasta sequence, not with respect to the coding sequence of the gene.

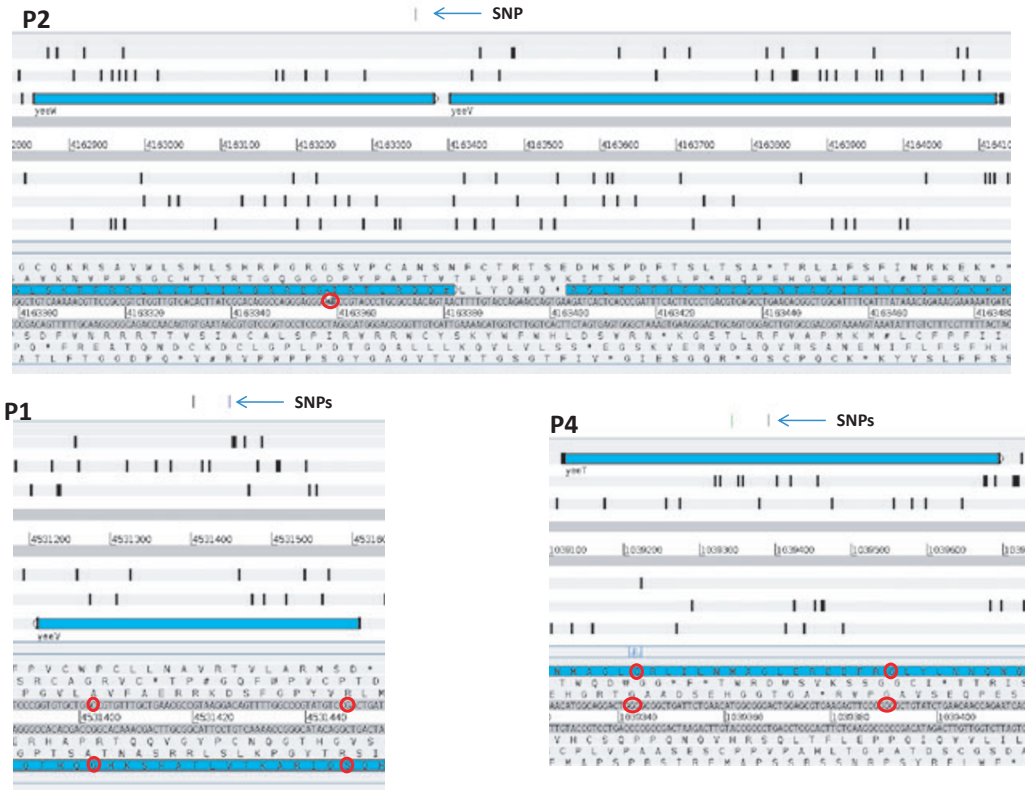

FIG. 2. ARTEMIS visualizations of the single nucleotide polymorphisms (SNPs) present in the blood isolates P1B (P1), P2B (P2) and P4B (P4) relative to their associated urine isolate. The SNPs are represented as lines (indicated by SNP and arrow) created by importing the VCF output file from the SAMTOOLS mapping pipeline into ARTEMIS. Their position relative to the coding sequence is denoted by the blue arrow representing the position of the coding sequences relative to the SNPs. The DNA sequence (grey) and protein encoding amino acid sequence (blue) is magnified and presented to show the position of the SNP and the resulting amino acid which is substituted (red circles).

revealed that these loci are present in all ExPEC in our strain collection.

Given that the genes associated with the minimum virulence factor requirement are commonly associated with various *E. coli* pathotypes we sought to determine if there were bacteraemia-specific factors in our five strains. To do this we determined the core genome of our five bacteraemia isolates by reciprocal BLAST searches [14] and then compared that to the reference genome of UTI89, which was isolated from uncomplicated cystitis [6] to allow identification of genes unique to the core bacteraemia gene set. Only one locus was present in our blood isolate genomes but not in the publically available genomes of *E. coli* associated with uncomplicated UTI or in *E. coli* K12 strains. The locus is a gene found in a ribose ABC transport system that is present in *Escherichia fergusonii* and is annotated as EFER_2514 to EFER_2523. Analysis of our

TABLE 3. Phenotypic comparison of matched isolates from patient urine and blood

Strain	Adhesion[a] (cfu/mL)	Invasion[a] (cfu/mL)	Biofilm[b] 37°C (A_{600})	Motility[c]	Serum resistance[d] T_0	T_3
P1U	3.1 E + 05	12	3.0	+	5.00E + 05	0
P1B	7.4 E + 04	33	2.3	+	3.83E + 06	6.50E + 06
P2U	6.6 E + 05	1 E + 04	3.0	+++	2.50E + 06	6.67E + 05
P2B	4.2 E + 05	2.2 E + 04	2.4	+++	3.00E + 06	2.33E + 06
P3U	2.8 E + 05	25	2.8	+++	3.50E + 06	5.17E + 05
P3B	1.5 E + 05	13	2.6	+++	1.83E + 06	1.67E + 07
P4U	2.2 E + 05	8.3 E + 03	3.5	+++	8.33E + 06	6.67E + 04
P4B	5.6 E + 06	1 E + 04	3.4	++	1.17E + 06	3.67E + 04

[a]Adhesion and invasion values represent CFU recovered from triplicate wells of infected T24 bladder epithelial cells *in vitro*. Assays were performed on three independent occasions and values are representative values of the repeated experiments.
[b]Biofilm assays were performed at 37°C in static cultures in both Luria Broth (LB) and brain–heart infusion broth in a 96-well plate, with five wells per strain. Assays were performed on three independent occasions and values are representative values of experiments repeated in LB broth.
[c]Motility assays were performed in duplicate on three independent occasions using semi sold LB agar incubated at 37°C. Values are semi-quantitative assessments of the levels of observed motility across the agar plates in each assay.
[d]Serum resistance was performed by the addition of quantified numbers of bacteria to human serum and enumeration of surviving bacteria after 3 h, and was performed in triplicate on two independent occasions.

annotated bacteraemia isolate genomes suggested that two (Patients 1 and 2) possess an identical operon situated in the identical location between the *entD* gene of the *fes/fep* operon and *ybdK*, with the other three carrying an orthologue of the EFER_2520 gene but in different locations. Comparison with the 15 available Phylogroup B2 ExPEC reference genomes on NCBI (http://www.ncbi.nlm.nih.gov/genome?term=escherichia%20coli) showed that the operon was absent in all but two strains, SE15 and NA114, both of which are isolates belonging to the highly pathogenic and drug-resistant ST131 lineage [15]. BLAST searches of the operon revealed that it is also present in isolates of *Klebsiella* spp. and the plant pathogens *Dickeya* spp. and *Pantoea* spp. but not in any other sequenced *E. coli*. We also screened our 150 bacteraemia and 150 UTI *E. coli* isolates by PCR for the gene EFER_2520. There was no definitive association of this island with sepsis with carriage rates of 31.4% in sepsis isolates and 27.2% in UTI isolates. However, there was clear association of carriage of this operon with the multidrug-resistant ST131 lineage, with 85% of isolates in our strain collection ($n = 39$) being PCR positive for the operon. This was not associated with extended spectrum β-lactamase carriage in ST131.

Discussion

From a clinical perspective the understanding of ascending urinary tract infection from bladder to kidney, and the resulting translocation of bacteria into the bloodstream, as the cause of urosepsis has been a well-defined process for many decades. What is less well understood, despite decades of fundamental research, is this process from the microbial perspective. At a microbial pathogenesis level almost all our understanding of the process of progression to urosepsis is based on classical bacterial genetics studies on ExPEC strains using model infection systems ranging from tissue culture cells to mouse infection models [16–18]. From a bacteriological perspective the confirmatory diagnosis of urosepsis is based on isolation of pure culture from the blood sample of a patient with preceding UTI, which is also usually based on pure culture; hence, the link between the two episodes is based purely on the presence of colonies of the same species in each sample.

The introduction of genome sequencing into clinical microbiology has challenged long-held clinical and bacteriological paradigms, ranging from how methicillin-resistant *S. aureus* and cholera have spread globally [19,20], to how methicillin-resistant *S. aureus* has emerged in hospitals at a local level [21]. Indeed, genome-scale investigations have unearthed variation and relatedness among pathogens missed by classical clinically utilized techniques [20] and because of their powerful level of discrimination are now being used in clinical disease investigations [22]. Given the fact that the association of ExPEC strains with urosepsis is based solely on the presence of *E. coli* on blood and urine culture plates, and that most of our perceived knowledge of the mechanisms of ascending UTI and urosepsis are based on surrogate models, we aimed to use the power of genomics to provide the most comprehensive study to date of urosepsis in human clinical cases.

Our analysis provided positive proof in four of our five sample sets that the same strain of *E. coli* can progress from UTI to bacteraemia in patients. More significantly we show that the classical ascending UTI virulence factor P pili is not present in all urosepsis isolates, and that there are just five loci associated with the bacteraemia phenotype. These are flagella, type 1 fimbriae, curli fimbriae, fes/fep iron acquisition system, and the cus heavy metal efflux system, all of which are commonly present across various *E. coli* pathotypes. Our analysis also shows that during the progression from UTI to bacteraemia there is little host imprint on the genome of the organism, with a difference of just a few SNPs between the blood and urine isolates and in one case no detectable

difference at all. Previous work on *E. coli* isolated from the human bladder over time showed that there was considerable host-specific imprint on the genome of the infectious agent [23]; however, these changes were in *E. coli* inhabiting the bladder over a prolonged time course. Our data suggests the rapid nature of transition from urinary tract to bloodstream results in very few selective changes in the organism. In three of the four matched isolates where SNPs were identified there was commonality in mutations occurring in the *yeeV* locus. This is a toxin/anti-toxin locus core to *E. coli* which has been suggested to play a role in cell shape and division [24,25]. The significance of these mutations warrants further investigation given that this is not a locus prone to mutation in long-term evolutionary studies performed in *E. coli* [26], and that the same locus was mutated in three of the four urosepsis cases. The amino acid substitutions described should result in large-scale changes to encoded proteins given the differences in molecular weight and properties in the amino acids as a result of the SNPs (Table 2); however, in the absence of characterization data for the proteins at this locus it is difficult to attribute a defined effect to those substitutions, although there is a clear correlation in our small data set between number of mutations in the locus and increased resistance to human serum as observed in P1U and P1B. This is currently the focus of investigation in our laboratory.

The data for our fifth patient also highlight the limitations of classical culture-based diagnosis of infectious disease. Despite a pure *E. coli* present on the urine and blood culture plate of patient 5, sequencing data clearly show the presence of at least three clearly distinct strains of *E. coli* in the patient. This has been seen before in familial outbreaks of enterohaemorrhagic *E. coli* [27] and in ExPEC wound infections [13]. Perhaps more importantly is the fact that there were two highly pathogenic ExPEC strain types in the urine, including an extended spectrum β-lactamase-positive ST131 strain, yet the causative agent of the bacteraemia in the patient was a completely antimicrobial-susceptible *E. coli* ST10 of phylogroup A origin.

In conclusion, we present genome-based data confirming the classically held urosepsis hypothesis as well as highlighting the true complexity of extra-intestinal *E. coli* infections. Our genome association studies also suggest that the vast majority of ExPEC found in nature have the genetic propensity to cause bacteraemia in humans.

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Transparency Declaration

The authors declare no conflicts of interest.

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