A Controlled Human Infection Model of Group A *Streptococcus* Pharyngitis: Which Strain and Why?

Joshua Osowicki,[a,b,c] Kristy I. Azzopardi,[a] Liam McIntyre,[d] Tania Rivera-Hernandez,[e] Cheryl-lynn Y. Ong,[e] Ciara Baker,[a] Christine M. Gillen,[e] Mark J. Walker,[e] Pierre R. Smeesters,[a,b,f,g] Mark R. Davies,[d] Andrew C. Steer[a,b,c]

[a]Tropical Diseases, Murdoch Children's Research Institute, Melbourne, Victoria, Australia
[b]Department of Paediatrics, University of Melbourne, Melbourne, Victoria, Australia
[c]Infectious Diseases Unit, Department of General Medicine, The Royal Children's Hospital Melbourne, Melbourne, Victoria, Australia
[d]Department of Microbiology and Immunology, The University of Melbourne, at the Peter Doherty Institute for Infection and Immunity, Melbourne, Victoria, Australia
[e]School of Chemistry and Molecular Biosciences and Australian Infectious Diseases Research Centre, The University of Queensland, St. Lucia, Queensland, Australia
[f]Paediatric Department, Academic Children Hospital Queen Fabiola, Université Libre de Bruxelles, Brussels, Belgium
[g]Molecular Bacteriology Laboratory, Université Libre de Bruxelles, Brussels, Belgium

ABSTRACT Group A *Streptococcus* (GAS) is a major cause of global infection-related morbidity and mortality. A modern controlled human infection model (CHIM) of GAS pharyngitis can accelerate vaccine development and pathogenesis research. A robust rationale for strain selection is central to meeting ethical, scientific, and regulatory requirements. Multifaceted characterization studies were done to compare a preferred candidate *emm*75 (M75) GAS strain to three other strains: an alternative candidate *emm*12 (M12) strain, an M1 strain used in 1970s pharyngitis CHIM studies (SS-496), and a representative (5448) of the globally disseminated M1T1 clone. A range of approaches were used to explore strain growth, adherence, invasion, delivery characteristics, short- and long-term viability, phylogeny, virulence factors, vaccine antigens, resistance to killing by human neutrophils, and lethality in a murine invasive model. The strains grew reliably in a medium without animal-derived components, were consistently transferred using a swab method simulating the CHIM protocol, remained viable at −80°C, and carried genes for most candidate vaccine antigens. Considering GAS molecular epidemiology, virulence factors, *in vitro* assays, and results from the murine model, the contemporary strains show a spectrum of virulence, with M75 appearing the least virulent and 5448 the most. The virulence profile of SS-496, used safely in 1970s CHIM studies, was similar to that of 5448 in the animal model and virulence gene carriage. The results of this multifaceted characterization confirm the M75 strain as an appropriate choice for initial deployment in the CHIM, with the aim of safely and successfully causing pharyngitis in healthy adult volunteers.

IMPORTANCE GAS (*Streptococcus pyogenes*) is a leading global cause of infection-related morbidity and mortality. A modern CHIM of GAS pharyngitis could help to accelerate vaccine development and drive pathogenesis research. Challenge strain selection is critical to the safety and success of any CHIM and especially so for an organism such as GAS, with its wide strain diversity and potential to cause severe life-threatening acute infections (e.g., toxic shock syndrome and necrotizing fasciitis) and postinfectious complications (e.g., acute rheumatic fever, rheumatic heart disease, and acute poststreptococcal glomerulonephritis). In this paper, we outline the rationale for selecting an *emm*75 strain for initial use in a GAS pharyngitis CHIM in healthy adult volunteers, drawing on the findings of a broad characterization effort spanning molecular epidemiology, *in vitro* assays, whole-genome sequencing, and animal model studies.

KEYWORDS *Streptococcus pyogenes*, controlled human infection model, group A *Streptococcus*, human challenge study, pharyngitis, vaccines

Address correspondence to Joshua Osowicki, joshua.osowicki@rch.org.au, or Andrew C. Steer, andrew.steer@rch.org.au.

Want to build a controlled human infection model? How will you choose which strain? Read how one group chose a GAS strain with the aim of safely and reliably causing pharyngitis in healthy adult volunteers. @JoshOsowicki

Group A *Streptococcus* (GAS; *Streptococcus pyogenes*) is a major contributor to global infection-related mortality and morbidity. It causes a diverse spectrum of human disease syndromes, from superficial infections (e.g., pharyngitis and impetigo) to invasive disease (e.g., necrotizing fasciitis and toxic shock syndrome) and autoimmune complications (acute rheumatic fever, rheumatic heart disease, and glomerulonephritis) (1). Development of a GAS vaccine has been impeded by scientific, regulatory, and commercial obstacles (2). Controlled human infection models (CHIM) are increasingly assuming an important role for vaccine development (3, 4). Drawing on the record of historical CHIM studies that included 172 participants (5–7), a modern pharyngitis CHIM in healthy adult volunteers has been proposed as part of a reenergized global effort to accelerate GAS vaccine development (8). Selection of a thoroughly characterized strain is central for development of a GAS CHIM.

A successful CHIM requires that infection and/or symptomatic disease endpoints are reached reliably and safely and bear sufficient resemblance to a natural state to suggest generalizability. The diverse clinical and microbiological profile of GAS presents challenges for CHIM study design, especially strain selection. There are more than 200 different GAS *emm* types. This widely used classification system is based on one part of the gene encoding a single GAS antigen, the M protein. No other antigen has been as closely studied, and the concept of M protein type-specific immunity has been a cornerstone of GAS research. GAS is a highly adapted human pathogen, and the limitations of *in vitro* assays and animal models have been well described. After more than a century of research, fundamental aspects of pathogenesis and human immune protection against GAS remain unknown. These knowledge gaps are simultaneously an argument for building a CHIM and a source of uncertainty in conceiving its design.

A thorough and explicitly stated rationale for strain selection is an important step in minimizing potential harm to participants and maximizing scientific impact. We considered desirable characteristics in selecting an initial strain to establish a GAS pharyngitis CHIM and surveyed available collections for suitable strains, focusing on an *emm*75 strain (GAS M75 611024, termed M75) isolated in 2011 from the throat of a 5-year-old girl with acute symptomatic pharyngitis in Melbourne (Table 1; see also Table S1 in the supplemental material) (9). In this paper, we present a multifaceted characterization of the preferred CHIM candidate M75 strain and compared it to three others: GAS M12 611025 (M12), an alternative challenge candidate; M1T1 5448 (5448), representative of the M1T1 clone recently responsible for most pharyngitis and invasive disease globally (10); and CDC SS-496 (SS-496), an M1 strain administered to 88 subjects in 1970s pharyngitis CHIM studies (5, 7).

RESULTS

Growth in an animal-free medium. Compared to that in THY broth, no detrimental effect on growth of M75, M12, and 5448 was observed in the animal-free medium (Fig. 1A). Eight-hour growth curves for M75 clones tested after 7 days of repeated *in vitro* passage were similar to those of the nonpassaged parent (data not shown).

Attachment properties. M75 had the highest adherence to D562 (75%) and HaCaT (81%) cells (Fig. 1B). M12 (53%) and 5448 (51%) were similarly adherent to D562 cells. M12 preferentially adhered to D562 over HaCaT cells (P = 0.005), whereas M75 and M1T1 showed no preference. The affinity of M12 to pharyngeal over skin cells matches its designation as an A-C pattern strain, associated with throat tissue tropism (11). Invasiveness of M75 and M12 was low for both cell lines ($\leq$0.45%). Invasion by 5448 of HaCaT cells (10%) was greater than that for D562 cells (0.2%) (Fig. 1B).

Capsule production. M75 produced 74 ng/ml of hyaluronic acid (HA) capsule, whereas M12 produced 7,506 ng/ml (Fig. 1C). Capsule production by 5448 matched previous findings (12).

Delivery characteristics and viability. The Dacron swab was considered most suitable for delivery of the challenge inoculum (Fig. S1). Mean broth uptake by Dacron (105 mg) and Rayon S (108 mg) swabs was comparable to that of cotton (129 mg), and

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TABLE 1 Preferred strain characteristics for a controlled human infection model of GAS pharyngitis[a]

Desirable strain characteristic(s)	Rationale	M75 611024 details
Definite but uncommon contemporary cause of symptomatic pharyngitis	Pharyngitis is the critical early target for GAS vaccine development; historical CHIM studies offer a template for a reliable and safe protocol; GAS pharyngitis is most common in childhood and adolescence, suggesting previous exposure and immune memory could prevent experimentally induced disease in adult volunteers	From a child with symptomatic GAS pharyngitis in Melbourne, 2011; preexisting immunity in adults is unknown (no correlate of protection); ≤5% of strains in most recent pharyngitis studies are *emm75*
Should cause skin infection	Common GAS skin infections (e.g., impetigo) will also be important in initial vaccine field trials; ideally, the pharyngitis CHIM strain(s) should also be suitable for use in a potential future human model of cutaneous GAS infection	E pattern generalist (throat and skin infections); cluster E6 is linked phylogenetically to D pattern skin isolates
Uncommon cause of invasive GAS disease and immunological sequelae	GAS pharyngitis can lead to locally invasive infectious complications (e.g., retropharyngeal abscess), severe invasive infection (e.g., streptococcal toxic shock syndrome), acute rheumatic fever, and glomerulonephritis	≤5% of isolates in recent reports of invasive GAS are *emm75*; from 2000 to 2016, 403/17,002 (2.4%) typeable invasive isolates reported to the U.S. CDC's Active Bacterial Core surveillance were *emm75* (Chris A. Van Beneden, personal communication, 11 September 2018) (41, 42); *emm75* strains rarely associated with ARF/RHD or APSGN (1)
Should have predictable and limited virulence and be suitable for use in animal models	Whole-genome sequencing, *in vitro* assays, and animal models may inform understanding of a GAS strain's relative virulence, although none fully predict human disease patterns	CovR/S virulence regulator, wild type (nonmutant); does not bind plasminogen and fibrinogen; *emm75* strains have been used in animal nasopharyngitis and invasive disease models
Should have limited antibiotic resistance	Ideally, the challenge strain should be eradicated from the pharynx by antibiotic treatment; resistance to penicillin has not been documented in GAS, but it does not reliably eradicate GAS from the pharynx; observed resistance to other drugs is variable	See the text
Challenge strain should possess a wide array of candidate vaccine antigens	For greatest impact, a GAS pharyngitis CHIM should be suitable for early use as a preliminary testing ground for vaccines	See the text

[a]See Table S1 for a detailed and referenced version of this table. ARF, acute rheumatic fever; APSGN, acute poststreptococcal glomerulonephritis; RHD, rheumatic heart disease.
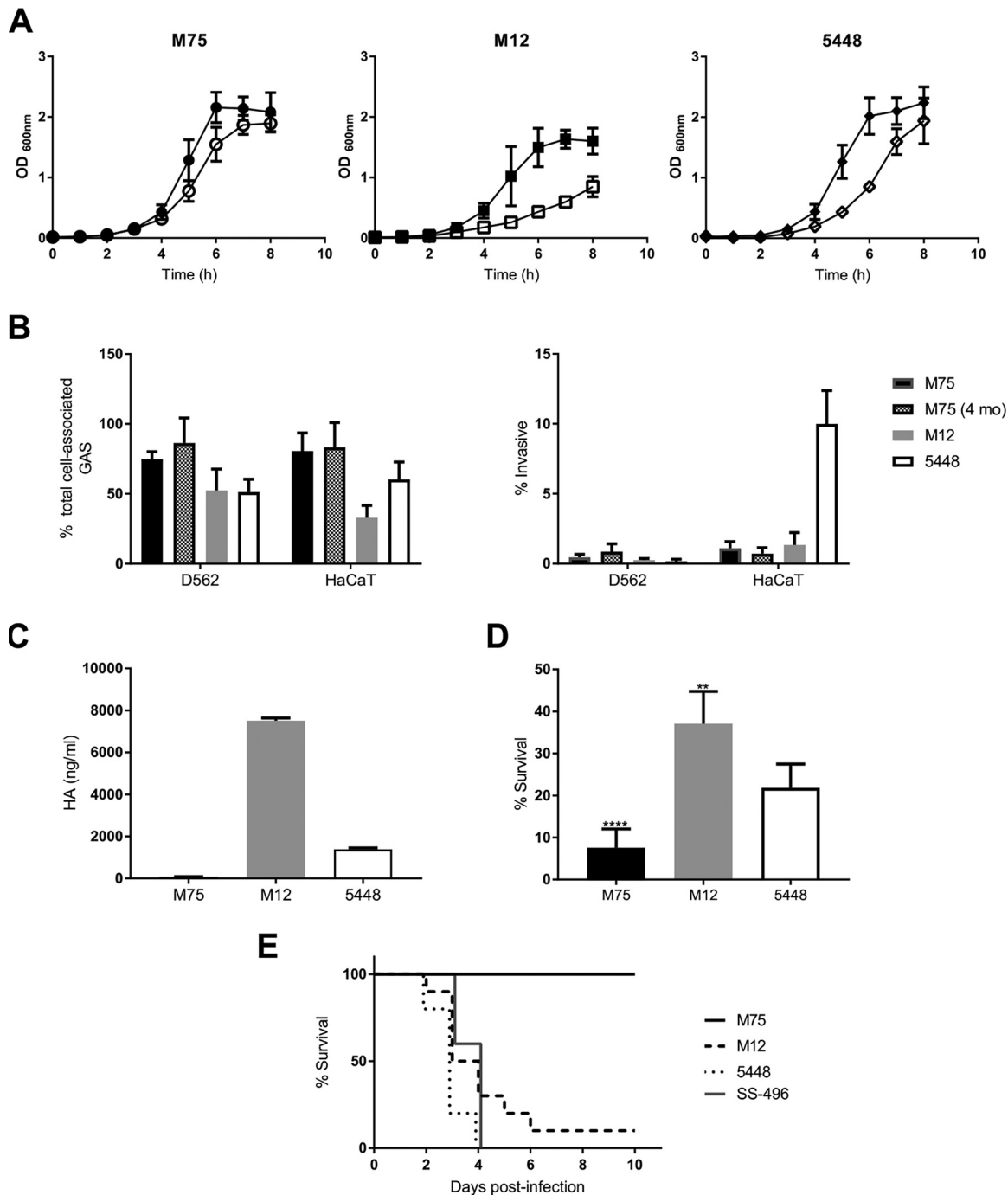
FIG 1 *In vitro* characterization of contemporary candidate strains for human challenge. (A) Growth kinetics of candidate strains in RPMI 1640 supplemented with 2% Veggietone (filled symbols) and Todd-Hewitt broth with 1% yeast extract (open symbols). Means and standard deviations (SD) are representative of three separate experiments done in triplicate. (B) Strain attachment and cellular invasion. Means and SD are from three separate experiments with triplicate wells. (C) Capsular hyaluronic acid quantification. Means and SD are derived from a single experiment. (D) Resistance of M75, M12, and 5448 to killing by human neutrophils. Means and SD are from three separate experiments using different blood donors, with seven biological replicates. (E) Strain lethality in a humanized plasminogen transgenic AlbPLG1 murine invasive disease model ($n = 10$ for each strain).

uptake variance was lowest (7.7 mg) for the Dacron swab. Superior release was noted for the Dacron swab with a mean of 1.8×10^3 CFU of M75 recovered after swab dipping (Fig. 2B). Recovery from M75 vials frozen for 4 months did not fall below 95% of the original inoculum at $T = 0$. Adherence and invasion were similarly unaffected by storage (data not shown).
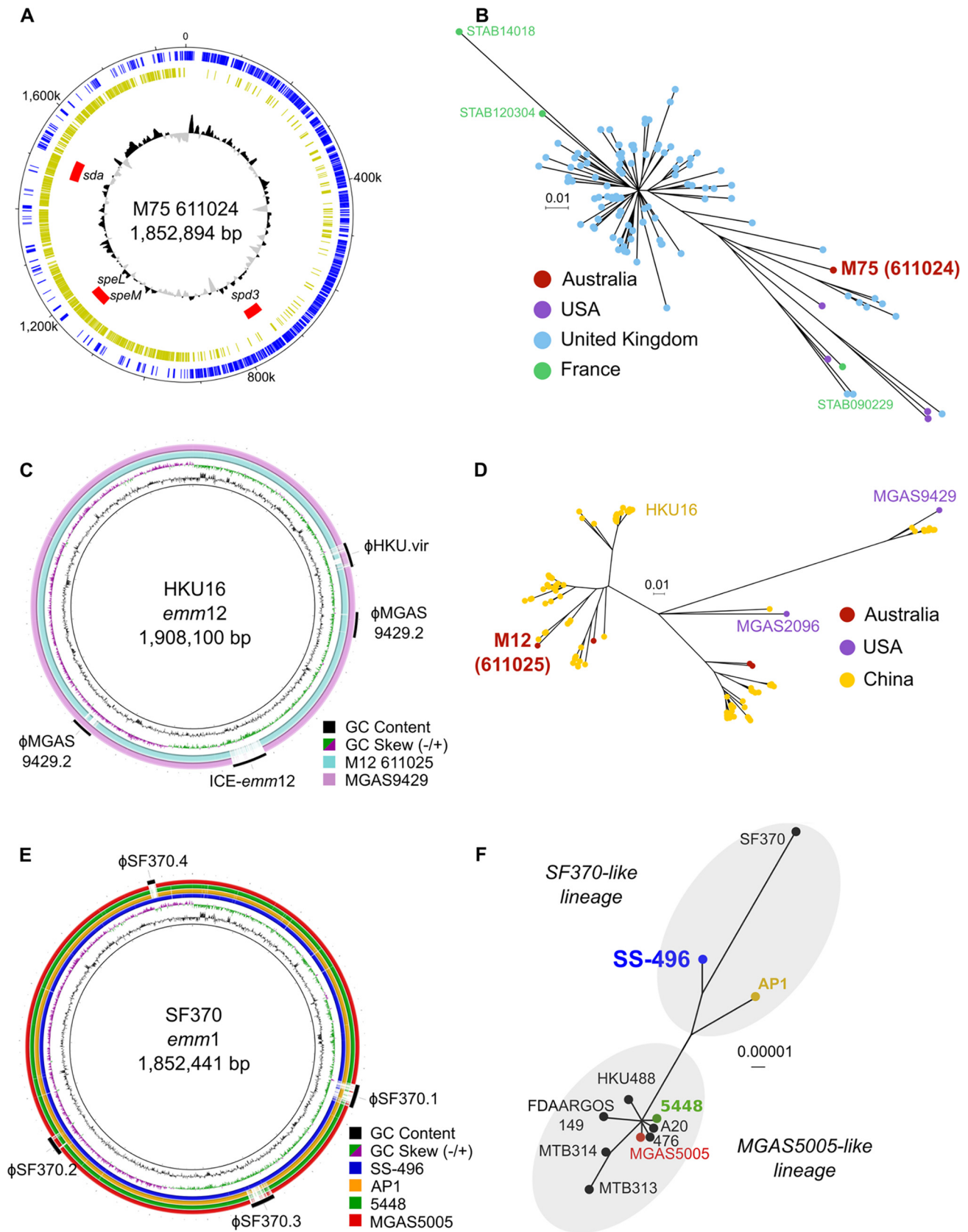
FIG 2 Comparative genomics of M75 611024, M12 611025, M1T1 5448, and M1 CDC SS-496. (A) Circular schematic of GAS M75 611024 showing GC plot (inner ring) with GC content above (black) and below (gray) the genome average. Predicted prophage sequences are shown in red, with associated prophage virulence determinants annotated and relative position of predicted coding sequences on the forward strand (blue) and reverse strand (gold).

TABLE 2 Antibiotic susceptibility of contemporary group A streptococcal strains M75 611024, M12 611025, and M1T1 5448

Antibiotic	Breakpoint[a] (mg/liter)			Etest MIC (mg/liter)		
	S	I	R	M75	M12	5448
Penicillin	≤0.12			0.012	0.016	0.012
Erythromycin	≤0.25	0.5	≥1	0.094	16	0.125
Clindamycin	≤0.25	0.5	≥1	0.125[b]	0.125[b]	0.125[b]
Azithromycin	≤0.5	1	≥2	1	64	1.5
Levofloxacin	≤2	4	≥8	0.5	4	0.5
Rifampin	≤0.06		>0.5	0.064	0.064	0.125

[a]All CLSI breakpoints except that for rifampin (EUCAST). I, intermediate susceptibility; R, resistant; S, susceptible.
[b]Inducible clindamycin resistance (D test) not detected.

Antibiotic susceptibility. M75 was susceptible to all tested antibiotics, while M12 was resistant to macrolides and fluoroquinolones (Table 2). All strains were susceptible to clindamycin, and inducible resistance was not detected.

Whole-genome sequencing and phylogenetic analyses. The complete genome of M75 611024 is comprised of a single chromosome of 1,852,894 bp (Fig. 2A). M75 has the multilocus sequence type (MLST) ST150 and contains the *emm*75.0 allele and *mrp*24 and *enn*334 alleles, corresponding to the *emm*-like genes *mrp* and *enn* (P. Smeesters, personal communication, July 2018). Three putative prophage sequences were identified in M75 harboring the endonuclease streptodornase 3 (*spd3*), pyrogenic exotoxins *speL* and *speM*, and the endonuclease *sdn*. M75 shared a hypothetical ancestral relationship with a UK *emm*75 cluster (Fig. 2B), yet it represents a distinct evolving lineage, suggesting an ancestral relationship to modern-day ST150 *emm*75 clones.

One single-nucleotide polymorphism (SNP) was found for each of three M75 clones sequenced after 7 days of repeated *in vitro* passage compared to sequence of the nonpassaged parent strain. Each SNP was intergenic and different, suggestive of random mutations of unlikely functional consequence (data not shown).

M12 611025 belongs to MLST ST36 and carries the *emm*12.0 allele. It shares a high degree of genome conservation with other *emm*12 genome sequences, varying in prophage and integrative conjugative element content relative to the reference genomes HKU16 and MGAS9429 (Fig. 2C). Phylogenetic analysis alongside 141 extant *emm*12 isolates showed an evolutionary relationship with other modern ST36 strains (Fig. 2D), including recent scarlet fever outbreak strains (13).

The historical challenge strain SS-496 shares a higher degree of genetic and evolutionary similarity with the ancestral M1 reference strain SF370 relative to the modern M1T1 strains MGAS5005 and 5448 (Fig. 2E and F) (14). SS-496 contains the historical SF370-like *purA* to *nadC* genomic region encoding streptolysin O.

Virulence factors and vaccine antigens. M75, M12, and SS-496 carry genes for an array of adhesion and invasion factors common to many *emm* types (Table 3). M75 contains a frameshift mutation in the fibronectin binding protein Sfb1 within the FCT locus. M12 carries the streptococcal superantigen A (*ssa*) gene recently reported in

FIG 2 Legend (Continued)
(B) Unrooted maximum likelihood tree of 131 *emm*75 strains from the United Kingdom, the United States, and France based on 1,046 SNPs relative to the M75 611024 reference genome. Tips of the tree are color coded based on country of isolation. Location of genomes corresponding to M75 611024 and the completely sequenced *emm*75 strains from France, STAB 090229 (CP020027), STAB 120304 (CP020082), and STAB 14018 (CP014542), are annotated. (C) Comparative BLASTN analysis of M12 611025 (blue ring) and MGAS9429 (purple ring) relative to the *emm*12 reference genome HKU16 (inner black circle). HKU16 GC content and GC skew are indicated in the inner ring, while annotated around the outside is the genomic position of known HKU16 mobile genetic elements. (D) Maximum likelihood phylogenetic relationship of strain 611025 with 141 *emm*12 *S. pyogenes* strains from other geographical regions based on 1,452 SNP sites from the core genome of the HKU16 reference genome. Tips of the tree are color coded based on country of isolation of each isolate. Genomes from completely sequenced *emm*12 strains MGAS9429 (CP000259), MGAS2096 (CP000261), and HKU16 (QMH11M0907901; AFRY01000001) are annotated. (E) Comparative BLASTN analysis of CDC SS-496 and other GAS M1 reference genomes, AP1, 5448, and MGAS5005, relative to the SF370 M1 GAS reference genome (inner black circle). (F) Mid-point-rooted maximum likelihood phylogenetic relationship of M1 GAS reference genomes based on 780 SNP sites. Tips of the tree are annotated by strain name and color coded by ring color from panel E. Genomes belonging to SF370-like and MGAS5005-like lineages (14) are clustered by gray shading. Comparative BLASTN analyses were generated using BRIG (40).

TABLE 3 Group A *Streptococcus* virulence factor genomic screen

Gene	Function	M75 611024	M12 611025	M1T1 5448	CDC SS-496
cfa-cfb	CAMP factor	✓	✓	✓	✓
tee (cpa)	T-pilus antigen		✓	✓	✓
cppA	Putative C3-degrading proteinase	✓	✓	✓	✓
emm	M-protein	✓	✓	✓	✓
endoS	Endo-beta-*N*-acetylglucosaminidase F2 precursor	✓	✓	✓	✓
fbp54	Fibrinogen-binding protein	✓	✓		✓
fctA	Major pilin Ap1 (FctA)			✓	✓
fctB	Minor pilin Ap2 (FctB)			✓	✓
grab	Protein G-related alpha 2M-binding protein		✓	✓	✓
hasA	HA synthase capsule	✓	✓	✓	✓
hasB	UDP-glucose 6-dehydrogenase capsule	✓	✓	✓	✓
hasC	Putative UDP-glucose pyrophosphorylase	✓	✓	✓	✓
htrA-degP	Serine protease	✓	✓	✓	✓
htsA	Putative ABC transporter periplasmic binding protein	✓	✓	✓	✓
htsB	Putative ABC transporter permease	✓	✓	✓	✓
htsC	Putative ABC transporter ATP-binding protein	✓	✓	✓	✓
hyl	Hyaluronoglucosaminidase	✓	✓	✓	✓
hylA	HA lyase precursor		✓	✓	✓
hylP	Hyaluronoglucosaminidase	✓	✓	✓	✓
ideS-mac	IgG-degrading protease	✓	✓	✓	✓
lepA	Signal peptidase I			✓	✓
lmb	Laminin binding protein	✓	✓	✓	✓
mf-spd	Deoxyribonuclease	✓	✓	✓	✓
mf3	Deoxyribonuclease	✓		✓	✓
plr-gapA	Glyceraldehyde-3-phosphate dehydrogenase	✓	✓	✓	✓
prtF2	Collagen adhesion protein		✓		
psaA	Manganese-binding protein	✓	✓	✓	✓
sagA	Streptolysin S precursor	✓	✓	✓	✓
sclA	Collagen-like surface protein A	✓	✓	✓	✓
sclB	Putative collagen-like protein			✓	✓
scpA	C5A peptidase precursor	✓	✓	✓	✓
sda	Phage-encoded streptodornase Sda		✓	✓	
sdn	Phage-encoded endonuclease Sdn	✓			
sfbII-sof	Fibronectin-binding protein	✓	✓		
sfbX	Fibronectin-binding protein	✓	✓		
shp	Hypothetical protein	✓	✓	✓	✓
shr	Fe^{3+}-siderophore transporter	✓	✓	✓	✓
sic	Streptococcal inhibitor of complement			✓	
ska	Streptokinase precursor	✓	✓	✓	✓
slo	Streptolysin O	✓	✓	✓	✓
smeZ	Enterotoxin	✓	✓	✓	✓
speB	Cysteine protease	✓	✓	✓	✓
speA	Exotoxin A			✓	
speG	Exotoxin G	✓	✓	✓	✓
speH	Exotoxin H		✓		✓
speI	Exotoxin I		✓		✓
speJ	Exotoxin J			✓	✓
speL	Exotoxin L	✓			
speM	Exotoxin M	✓			
spyA	C3 family ADP-ribosyltransferase	✓	✓	✓	✓
srtC1	Sortase			✓	✓
ssa	Streptococcal superantigen A		✓		
tig-ropA	Trigger factor	✓	✓	✓	✓

scarlet fever-associated isolates in China and the United Kingdom (15). M12 does not carry the multidrug-resistant integrative conjugative element ICE-*emm*12 or the *ssa*-carrying prophage ΦHKU.vir, linked to the emergence of scarlet fever clades (13). The virulence profile of SS-496 is similar to that of pre-1980 M1 strains such as SF370, with *speH* and *speI* exotoxins and the absence of the *speA* exotoxin typical of modern isolates such as 5448 (Table 2). M75, M12, SS-496, and 5448 all possess wild-type *covRS* and *ropB* two-component virulence regulators.

High carriage (>60%) of protein and peptide candidate vaccine antigens was observed for M75 and M12 using a homology-based genome approach (Table 4).

TABLE 4 Group A *Streptococcus* candidate vaccine antigen genomic screen[c]

Gene/antigen	Gene identifier[a]	Function	M75 611024	M12 611025	M1T1 5448
		M-protein, N terminal (30-valent vaccine)	✓	✓	✓
		M-protein, C terminal (J8.0)		✓	✓
		M-protein, C terminal (StreptInCor T-cell epitope)			
		M-protein, C terminal (StreptInCor B-cell epitope)			
		M-protein, C terminal (StreptInCor common epitope)		✓	
adi	MGAS5005_spy1275	Arginine deaminase	✓	✓	✓
fbaA	MGAS5005_spy1714	Fibronectin-binding protein A			
fbp54	AAA57236	Fibronectin-binding protein 54	✓	✓	✓
oppA	M5005_spy0249	Oligopeptide-binding protein	✓	✓	✓
GAC	MGAS5005[b]	Group A carbohydrate	✓	✓	✓
pulA	SF370_spy1972	Putative pullulanase	✓	✓	✓
r28	AF091393	Rib-like cell wall protein			
scpA	MGAS5005_spy1715	C5a peptidase	✓	✓	✓
sfbI	X67947	Streptococcal fibronectin binding protein I			
sfbII-sof	X83303	Serum opacity factor	✓		
shr	SPY1530	Streptococcal hemoprotein receptor	✓	✓	✓
sib35	AB254157	Streptococcal immunoglobulin-binding protein 35	✓	✓	✓
slo	M5005_spy0124	Streptolysin O	✓	✓	✓
spa	MGAS8232_spyM18_2046	Streptococcal protective antigen			
speA	X03929	Streptococcal pyrogenic exotoxin A			✓
speB	M5005_spy1735	Cysteine protease	✓	✓	✓
speC	SF370_spy0711	Streptococcal pyrogenic exotoxin C			
spy0651	MGAS5005_spy0651	Cell surface protein	✓	✓	✓
spy0762	MGAS5005_spy0762	Hypothetical membrane associated protein	✓	✓	✓
spy0942	MGAS5005_spy0942	Nucleoside-binding protein	✓	✓	✓
spyAD	MGAS5005_spy0229	Adhesin and division protein	✓	✓	✓
spyCEP	MGAS5005_spy0341	Interleukin-8 serine protease	✓	✓	✓
sse	SF370_spy1407	Serine esterase		✓	✓
tee	MGAS5005_spy0109	T antigen	✓	✓	✓
tif	SF370_spy1612	Trigger factor	✓	✓	✓

[a]Nucleotide gene sequences derived from completely sequenced genomes or listed GenBank identifiers. Accession numbers for genome sequences include MGAS5005 (CP000017), SF370 (AE004092), and MGAS8232 (AE009949).
[b]GAC operon (~14.2 kb) refers to MGAS5005 genome coordinates 604873 to 619151.
[c]BLAST analyses at a homology level of 80% for protein antigens and 100% for peptide-derived sequences.

Human neutrophil killing assay. M75 was most susceptible to *in vitro* killing when incubated with human neutrophils, although killing was observed for all strains (Fig. 1D).

Mouse lethal invasive model. Compared to M12, 5448, and SS-496, M75 was avirulent in the humanized mouse invasive disease model (Fig. 1E).

DISCUSSION

We have described the rationale for selecting M75 for initial use in a new GAS pharyngitis CHIM in healthy adults, including results of diverse preclinical studies assessing its fitness for purpose. For context and comparison, we have presented results for three other strains (M12, 5448, and SS-496).

M75 is compatible with critical protocol points: reliable growth in an animal-free medium, retention of growth and attachment properties after prolonged storage at −80°C, consistent delivery using a commercially available swab, and susceptibility to antibiotics used to treat GAS pharyngitis. M75 looks to have an acceptable virulence profile, with the capacity to cause pharyngitis and low potential for invasive disease. M75 has attractive attachment properties for immortalized human pharyngeal and skin cell lines, with limited cellular invasion. M75 was highly susceptible to *in vitro* killing by human neutrophils, possibly due to its minimal capsular HA production. In a humanized mouse model of invasive infection M75 was avirulent, whereas M12 and both M1 strains were lethal. Whole-genome sequencing placed the strains in the context of epidemiologically related phylotypes and found broad representation of candidate vaccine antigens and a relatively restricted array of virulence factor genes in M75.

CHIM strain selection has been guided by varied general and pathogen-specific considerations, all with the goal of safely and reliably reproducing relevant and

generalizable asymptomatic (infection/carriage) or symptomatic (disease) study endpoints (3, 4, 16). Suitable well-characterized strains may already exist, such as the *Salmonella enterica* serovar Typhi Quailes strain (17). Patients with mild to moderately severe uncomplicated disease may be a source of naturally attenuated new strains. Multiple strains, sometimes from different locations, may be required to represent natural strain diversity and/or enable heterologous rechallenge (18). If mechanisms of severe infection and/or complications are known, the implicated virulence factor(s) may be avoided (e.g., Shiga toxin-producing *Escherichia coli* [19] and *Campylobacter jejuni* inducing cross-reactive antibodies to GM1 and GQ1b gangliosides [20]). Pathogens may be modified for use, such as the propagation of single-sex *Schistosoma mansoni* cercariae to prevent chronic schistosomiasis (21). For vaccine studies, target antigen(s) must be present in the challenge strain(s). In every instance, strains must be characterized and be compatible with protocols for manufacturing and inoculation and with techniques to measure organism and host responses.

The limitations of this characterization effort are inherent in the rationale for pursuing a GAS CHIM. *In vitro* assays, genomics, and animal models do not fully capture or predict the dynamic elements and sequelae of human infection by GAS, a highly adapted and human-restricted pathogen. Even advanced nonhuman primate models produce a pharyngitis syndrome with important differences from human disease. A single contemporary clone, represented here by 5448, is simultaneously the most common cause in urbanized settings of both the mildest and most severe disease syndromes, with the basis for tissue tropism and bacterial-human genotype-phenotype relationships still relatively obscure. These uncertainties dictate a cautious approach extending beyond strain selection, including strain manufacture following principles of Good Manufacturing Practice, initial inclusion of healthy adults only without risk factors for severe GAS disease, a dose-ranging study to establish attack rate and safety, inpatient admission at a trials facility supported by a tertiary hospital, universal antibiotic treatment, outpatient follow-up, and echocardiography at screening and final visits.

A generic limitation of CHIM studies is the uncertain degree to which data from healthy adults experiencing a single syndrome (pharyngitis) caused by one strain (M75) can be generalized to other subjects, syndromes, strains, and settings (e.g., children with GAS skin infections due to other *emm* types in low- and middle-income countries). While inclusion of other strains and even a skin infection CHIM are conceivable extensions, model findings must be interpreted alongside knowledge derived from more naturalistic studies. For vaccine development, a GAS pharyngitis CHIM has dual scientific and strategic purposes, aiming to serve as a bridge to field trials with a more natural distribution of subjects, syndromes, and strains.

With a view to the very high priority given to participant safety and risk minimization, findings from these strain characterization studies reinforce the appropriateness of M75 for initial use in a GAS pharyngitis CHIM.

MATERIALS AND METHODS

Bacterial isolates. M75 611024 and M12 611025 were isolated in 2011 from throat swabs collected from children with acute pharyngitis in Melbourne, Australia, and stored at the Murdoch Children's Research Institute (9). Mark Walker at the University of Queensland supplied 5448 (10, 22). The U.S. Centers for Disease Control and Prevention (CDC) *Streptococcus* Laboratory provided the SS-496 strain, submitted in 1958 from Duke University.

Growth and viability. For administration to human volunteers, an animal-free medium must sustain sufficient strain growth. A chemically defined medium was developed (VR broth) consisting of RPMI 1640 (Gibco) and 2% (wt/vol) Veggietone genetically modified organism-free soya-peptone (Oxoid). Eight-hour growth assays were done comparing growth of M75 in this medium to that in Todd-Hewitt broth (Oxoid) with 1% (wt/vol) yeast extract (Bacto) (THY). Bacteria were grown in 125-ml Erlenmeyer flasks containing 25 ml of VR or THY broth and agitated gently at 75 rpm. To simulate manufacturing processes, M75 growth in VR broth was examined after 7 days of repeated *in vitro* passage, using frozen cultures of three postpassage clones and the prepassage parent isolate.

HA capsule assay. The hyaluronic acid (HA) capsule is a GAS virulence factor that resists opsonophagocytosis (23). Capsular HA levels were quantified using a test kit (Corgenix), as previously described (10).

Attachment properties. Cell culture lines have been used to study GAS adherence (24, 25). We used Detroit 562 (D562) human pharyngeal cells and skin HaCaT cells, simulating natural sites of infection. As previously described, total cell-associated GAS (percentage of original inoculum) and invasiveness (intracellular fraction of total cell-associated GAS) were determined using GAS grown to mid-exponential phase (optical density at 600 nm [OD_{600}] of ~0.5) in VR broth and diluted in 500 μl of assay medium (MEM with 5% fetal bovine serum; Gibco) to a multiplicity of infection of 5:1 (GAS:cells) (10). Inoculated trays were centrifuged for 5 min at 200 × g and incubated for 1 h at 37°C in 5% CO_2 and then washed three times with phosphate-buffered saline (PBS) to remove nonadherent bacteria. Cell-associated GAS (adherent plus invasive) were detached using 200 μl 0.25% trypsin, lysed with 0.025% Triton-X (Sigma) in distilled water, and enumerated by track dilution on horse blood agar. To measure invasive bacteria, cells were washed once after incubation in assay medium for 1 h and then incubated for another hour in medium containing 100 μg/ml gentamicin and enumerated as before.

Delivery characteristics. To assess M75 viability following storage at −80°C, bacteria were grown in VR broth (OD_{600} of 0.5), centrifuged, and suspended in broth containing 10% (vol/vol) glycerol (Sanofi). Vials containing 10^5, 10^6, 10^7, and 10^8 CFU/ml were thawed at intervals and immediately tested without washing (mimicking the challenge protocol) for (i) growth in solid and liquid media, (ii) viability by enumeration, and (iii) attachment properties, as described above.

For the challenge procedure, swab uptake and release of the GAS inoculum should be consistent. We simulated direct oropharyngeal application using four Copan swabs: FLOQSwab (nylon), Dacron (polyester), and small (S) and large (L) rayon swabs. For uptake, vials of broth were weighed before and after dipping of swabs for 10 s. Swab release of GAS was measured by dipping swabs in 1-ml vials containing 1×10^5 to 3×10^5 CFU of M75 for 10 s, followed by transferring to 1 ml of PBS for 10 s and then enumerated by spread plate dilutions.

Antibiotic susceptibility testing. MICs were determined by Etest, and double disk diffusion (d-zone test) was used to detect inducible clindamycin resistance. Interpretive breakpoints of the Clinical and Laboratory Standards Institute (CLSI; penicillin, erythromycin, azithromycin, clindamycin, and levofloxacin) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST; rifampin) were used (26, 27).

Whole-genome sequencing and phylogenetic analyses. The complete M75 611024 genome sequence was determined using long-read single-molecule real-time sequencing on the Pacific Biosciences RS II platform. Filtering of the long reads identified 104,694 reads with an average polymerase read length of 4.1 kb. A single circular assembly was generated using SMRT analysis, v2.3.0 (Pacific Biosciences), and HGAP, v3, and polished using Quiver at an average read depth of 96-fold. To aid in assembly validation, M75 was also sequenced on an Illumina Next-seq 500 to produce paired-end reads with a read length of 150 bases. The M75 611024 genome sequence has been submitted to GenBank (accession number CP033621). The genomes of M12 611025 and CDC SS-496 were sequenced by Illumina Next-seq 500 with a paired-end read length of 150 bases. Draft genome assemblies were generated using SPAdes v3.12.0. Illumina short reads of M12 611025 (accession number SRR8217179) and CDC SS-496 (SRR8217180) have been submitted to the Short Read Archive (PRJNA504701).

To study M75 genomic stability, three clones were sequenced by Illumina Next-seq 500, with 150-bp paired-end reads, after 7 days of repeated *in vitro* passage. These sequences were aligned with the prepassage parent M75 reference sequence to identify single-nucleotide polymorphisms (SNPs).

Phylogenetic analysis of a global data set of *emm*75 isolates was determined by mapping short read sequences of 131 global *emm*75 genomes from the United Kingdom ($n = 124$), United States ($n = 4$), and France ($n = 3$) (28–30) to the M75 611024 reference genome with BWA MEM (v0.7.16). SNPs with a Phred quality score of ≥30 were identified in each isolate using SAMtools pileup with a minimum coverage of 30×. Prophage sequences within M75 611024 were identified using the Phaster server, with SNPs located within these prophage excluded, as they represent evolutionary confounders. A maximum likelihood phylogenetic tree was built from 1,046 concatenated SNP sites using RAxML, v8.2.8, with the general time-reversible model and gamma correction with 100 bootstrap resamplings to assess phylogenetic support.

Phylogeny of 141 *emm*12 genomes, including the Illumina reads of M12 611025 and sequences from Australia, the United States, Hong Kong, and mainland China, was analyzed by mapping to the reference genome HKU16 (strain QMH11M0907901 [GenBank accession no. AFRY01000001]) from 1,452 vertically inherited SNPs as previously described (13). Illumina reads of the M1 genome sequence CDC SS-496 were mapped to MGAS5005 (GenBank accession no. NC_007297) and other M1 reference genomes with phylogeny inferred form 780 vertically inherited SNPs.

Virulence factors and vaccine antigens. Virulence gene carriage was determined for M75, M12, 5448, and SS-496 by blastN screening assemblies against the virulence factor database (VFDB) (31). Gene presence was defined by an 80% nucleotide cutoff over 80% of the gene length.

Protection in animal models has been shown for more than twenty-five candidate GAS vaccine protein antigens and several peptide-based antigens (32). For protein antigens, presence was defined by an 80% nucleotide cutoff over 80% of the gene length. For sequence-constrained peptide-based vaccine epitopes J8.0 (SREAKKQVEKAL) (33) as well as the StreptInCor sequence (KGLRRDLDASREAKKQLEAEQQ KLEEQNKISEASRKGLRRDLDASREAKKQVEKA) (34) and associated T-cell (KGLRRDLDASREAKKQLEAEQQ), B-cell (ASRKGLRRDLDASREAKKQVEKA), and common B-T-cell (KGLRRDLDASREAKKQ) epitopes, a 100% nucleotide sequence match was taken to define presence, although 100% homology may not be required to induce production of broadly cross-reactive antibodies and vaccine protection.

Neutrophil killing assay. Survival of M75, M12, and 5448 incubated with human neutrophils *in vitro* was assayed as previously described (35). Experiments were performed in triplicate using mid-

exponential-phase GAS at a multiplicity of infection of 10:1. Differences in neutrophil survival were analyzed using 1-way analysis of variance (GraphPad Prism).

Murine invasive model. Strain virulence was compared in a humanized plasminogen transgenic *AlbPLG1* mouse model (36). In separate experiments, groups (*n* = 10) of *AlbPLG1*$^{+/-}$ mice were administered subcutaneous doses of either M75 (7×10^7 CFU), M12 (8×10^7 CFU), 5448 (5×10^7 CFU), or SS-496 (3×10^7 CFU), and survival was monitored for 10 days, as previously described (37, 38).

Ethics statement. Animal procedures followed the *Australian Code for the Care and Use of Animals for Scientific Purposes* and were approved by the University of Queensland Animal Ethics Committee (SCMB/140/16/NHMRC) (39). An initial dose-ranging CHIM study has been approved by The Alfred Hospital Ethics Committee (500/17) and is registered at ClinicalTrials.gov (NCT03361163).

Data availability. The M75 611024 genome sequence has been submitted to GenBank (accession number CP033621). Illumina short reads of M12 611025 (accession number SRR8217179) and CDC SS-496 (SRR8217180) have been submitted to the Short Read Archive (PRJNA504701). M75 was also sequenced on an Illumina Next-seq 500 to produce paired-end reads with a read length of 150 bases (accession number SRR8217178).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/mSphere.00647-18.

FIG S1, PDF file, 0.1 MB.

TABLE S1, PDF file, 0.2 MB.

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The lead author affirms that the manuscript is an honest, accurate, and transparent account of the study being reported, that no important aspects of the study have been omitted, and that any discrepancies from the study as planned have been explained. We have no financial or other conflicts to declare.

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