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RAPID COMMUNICATION

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TLR-2 gene *Arg753Gln* polymorphism is strongly associated with acute rheumatic fever in children

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Abstract The recently described family of toll-like receptors (TLRs) is a key player in host immunity by mediating inflammatory reactions against a wide range of pathogens. Mutations and polymorphisms in TLRs have revealed the importance of TLRs in human defence against diseases. TLR-2 is reported to interact with different bacterial structures, including lipoproteins, peptidoglycan and lipoteichoic acid. To assess the role of TLR-2 gene polymorphism in acute rheumatic fever (ARF) etiopathology, 61 independent Caucasian Turkish patients and 91 child and 116 adult controls were studied. Antistreptolycin O, C-reactive protein, sedimentation and white blood cell counts were studied to evaluate the clinical characteristics of the patients. Genomic DNA was extracted from peripheral blood using a standard column extraction technique. The *Arg753Gln* and *Arg677Trp* polymorphisms were genotyped by polymerase chain reaction (PCR) restriction fragment length polymorphism. The PCR products for the TLR-2 gene were analysed on 1.5% agarose gel pre-stained with ethidium bromide. Compared with healthy adult controls, the *Arg753Arg* genotype was significantly decreased in the entire group of ARF cases [odds ratio (OR) 0.01, 95% confidence interval (95% CI) 0.0034–0.031, $p<0.0001$]. Significantly, ARF patients were just 16 times more frequent with *Gln* allele (OR 15.6, 95% CI 7.87–30.8, $p<0.0001$). Moreover, evidence for an intensifying effect of the *Gln* allele was noteworthy when patients with *Arg753Gln* genotype were compared with healthy controls (OR 97.1, 95% CI 32.5–290, $p<0.0001$). However, no *Arg677Trp* polymorphism was detected in either patients or controls. Our data suggest that there is strong evidence for the biological role of TLR-2 in ARF. The common TLR-2 *Arg* to *Gln* polymorphism at position 753 significantly contributes to the pathogenesis of ARF. These results will allow the construction of a profile of individuals prone to ARF and may assist in developing new therapies.

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Abbreviations ARF: Acute rheumatic fever · RHD: Rheumatic heart disease · TLR: Toll-like receptor · PCR: Polymerase chain reaction · RFLP: Restriction fragment length polymorphism · ASO: Antistreptolysin O · CRP: C-reactive protein · ESR: Erythrocyte sedimentation rate · WBC: White blood cells count · NF-κB: Nuclear transcription factor kappa B

Introduction

Acute rheumatic fever (ARF) and rheumatic heart disease (RHD) are non-suppurative complications of group A streptococcal pharyngitis due to a delayed immune response. Group A streptococci are the most common bacterial causes of pharyngitis in both developing and developed countries, with a peak incidence in children 5–15 years of age [1–4]. It has been estimated that most children develop at least one episode of pharyngitis per year, 15–20% of which are caused by group A streptococci [2–5]. Despite a documented decrease in the incidence of ARF and a similar documented decrease in the prevalence of RHD in industrialized countries during the past five decades, these non-suppurative cardiovascular sequels of group A streptococcal pharyngitis remain to be medical and public health problems in both industrialized and industrializing countries, even at the beginning of the twenty-first century [5–8]. Turkey is one of the countries where the incidence of ARF and, consequently, the prevalence of RHD have declined remarkably over the last decades but are still high especially in low socio-economic groups [9, 10]. Only pharyngitis caused by group A streptococci has been closely linked with the etiopathogenesis of ARF and RHD. The economic effects of the disability and premature death caused by these diseases are felt at both the individual and national levels through higher direct and indirect health-care costs. The most devastating effects are on children and young adults in their most productive years.

Several studies were done to identify the genetic factors of susceptibility in ARF. Previous studies have reported an association with human leucocyte androgen (HLA)-B27, HLA-DR antigens and transforming growth factor-β 1 gene polymorphisms in ARF and RHD [11–18]. In our previous studies, we demonstrated that there was an association between the FcγRIIA 131R allele polymorphism [19] and MIF gene C allele polymorphism at position 173 in the promoter region and predisposition to ARF in children (unpublished data). A central feature of innate immunity is that it targets microbial components that possess structures essential for the survival of the organism. It has recently been established that cell-wall components of bacterial pathogens are recognized by the toll-like receptor (TLR) [20, 21].

Toll-like receptors are sensors of foreign microbial products, which initiate host defence responses in all multicellular organisms. TLRs can initiate the entire host defence, be it innate or adaptive [22]. Mouse and human studies have demonstrated the importance of TLR signalling in host defence. In addition, vast arrays of microbial molecules have been shown to stimulate TLRs [23–25]. Mice lacking TLR-2 are more susceptible to infection with various gram-positive bacteria [25, 26]. Moreover, mutations and polymorphisms in TLRs and TLR signalling molecules have revealed the importance of TLRs in human defence against pathogens [27]. A polymorphism of the TLR-2 gene has been described, leading to the replacement of arginine by glutamine at position 753, and initial investigations by single-strand conformational polymorphism provided evidence for a correlation of the polymorphism with the incidence of sepsis caused by gram-positive bacteria in humans [28]. There are also examples emerging of polymorphisms in TLR-4, TLR-5 and IRAK-4 being associated with diseases [23, 24, 29–31]. Cells from these patients are unresponsive to a wide range of TLR stimuli, and the patients are overwhelmed by a variety of childhood infections. Recently, Hawn et al. identified a common polymorphism in TLR-5 that encodes a stop codon and renders cells from these individuals hyporesponsive to bacterial flagellin [29].

These data provide evidence that polymorphism of the TLR-2 gene may lead to increased susceptibility to infections by bacteria containing TLR-2 agonists, such as gram-positive group A streptococci, and assessment of polymorphism in patients with ARF may lead to valuable information about risk stratification. Here, we studied the possible influence of TLR-2 polymorphisms, which might be useful in contributing to the identification of the primary risk factors associated with the pathogenesis of ARF.

Materials and methods

Study patients and control population

The study consisted of 61 Caucasian Turkish ARF patients (31 male and 30 female; ages 11.16±2.88, at admission for ARF diagnosis) followed up in the pediatric clinic of the Ege University School of Medicine (Izmir, Turkey) who fulfilled the revised classification criteria of Jones [32]. Ninety-one ethnically matched, unrelated, healthy children (50 boys, 41 girls; ages 8.71±1.3) and 116 ethnically matched, unrelated, healthy adult volunteers (54 men, 62 women; ages 28.04±6.6) were also included in this study. Arthritis was associated with ARF in 21 patients (34.4%), carditis in 18 patients (29.5%), arthritis and carditis in 17 patients (27.9%), carditis and chorea in 3 patients (4.9%) and chorea in 2 patients (3.3%). Patients did not have any other systemic disease. Control groups were selected both from children and adults to exclude persons who might have a genetic risk for ARF in childhood. No medical or family history of any systemic or autoimmune disease has been reported in the control groups. The procedures were in accordance with the ethical standard for human experi-

mentation established by the Declaration of Helsinki of 1975, which was revised in 1983.

Clinical measurements

All patients were asked to fast for at least 12 h and to avoid smoking and heavy physical activities for at least 2 h before examination. Blood was collected with minimal stasis during admission to the clinic with ARF pre-diagnosis. Antistreptolysin O (ASO) and C-reactive protein (CRP) were measured colorimetrically on an Olympus AU 2700 chemical analyser. Erythrocyte sedimentation rate (ESR) was measured by the Westergren method. White blood cells count (WBC) was performed with a Coulter counter.

Genomic DNA preparation and quantitation

Genomic DNA was extracted from ethylenediaminetetraacetic acid (EDTA) anticoagulant whole blood samples employing the QIAmp blood DNA mini-kit (Qiagen, Hilden, Germany) following manufacturer's instructions. DNA concentration was determined using the PicoGreen dsDNA quantitation kit (Molecular Probes Inc., Eugene, OR, USA) according to manufacturer's instructions.

Polymerase chain reaction and enzyme digest

TLR-2 gene *Arg753Gln* and *Arg677Trp* polymorphisms were genotyped by the method which Schröder et al. previously reported [33]. Both TLR-2 polymorphisms led to the elimination of a restriction site for *Aci*I, with the *Arg753Gln* polymorphism creating a novel site for *Sfc*I at 2265 bp. Designed primers spanned a region of 340 bp, including both polymorphisms, using the following primers: forward 5′-GCCTACTGGGTGGAGAACCT-3′ and reverse 5′-GGCCACTCCAGGTAGGTCTT-3′. For investigation of *Arg753Gln* polymorphism, only an additional forward primer, 5′-GGGACTTCATT-CCTGGCAAGT-3′, was designed, yielding a 264-bp product.

Amplification was carried out on a GeneAmp PCR System 9700 (PE Applied Biosystems, Foster City, CA, USA) in a 25-μl reaction mixture in 0.2-ml thin-wall polymerase chain reaction (PCR) strip tubes (Axygen Scientific, Inc., CA, USA) containing 1 μl genomic DNA solution; GeneAmp Gold Buffer (5 mmol/l Tris-HCl, pH 8.0, 50 mmol/l KCl; PE Applied Biosystems); 2.0 mmol $MgCl_2$; 50 μmol/l each of the dGTP, dATp, dTTP and dCTP (Promega Inc., Madison, WI, USA); 25 pmol each forward and reverse primers and 1.0 U AmpliTaq Gold polymerase (PE Applied Biosystems). The cycling conditions comprised a hot start at 95°C for 10 min, followed by 35 amplification cycles at 95°C for 30 s, 58°C for 30 s and 72°C for 25 s, followed by one elongation step at 72°C for 5 min. Three microlitres of the PCR product was incubated for 2 h with 0.5 U *Aci*I enzyme (New England Biolabs, Beverly, MA, USA) in a total volume of 10 μl at 36°C.

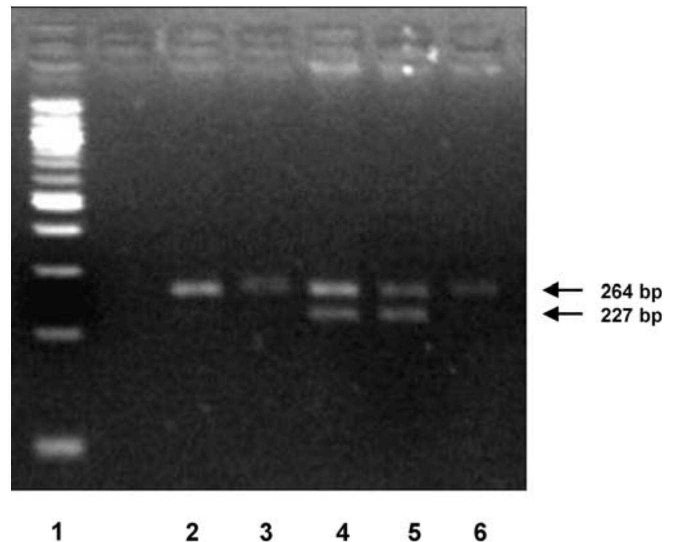

Fig. 1 Agarose gel electrophoresis of PCR products for TLR-2 gene in patients and control subjects. Photograph of UV-illuminated DNA resolved on 3.0% gels by electrophoresis, stained with ethidium bromide. *Lane 1* is 100 bp DNA ladder (MBI Fermentas, Vilnius, Lithuania). *Lane 2*, *Lane 3* and *Lane 6* are *Arg753Arg* homozygotes. *Lane 4* and *lane 5* are *Arg753Gln* heterozygotes. Note that the characteristic of DNA fragmentation in allele *Arg* yielded a fragment of 264 bp, whereas allele *Gln* yielded a 227-bp fragment

Samples were subjected to electrophoresis on gels containing a mixture of 1.5% agarose (Sigma, Deisenhofen, Germany) pre-stained with ethidium bromide and 1.5% NuSieve GTG (BMA, Rockland, ME, USA) and run for 1.5 h at 70 V (Fig. 1).

Furthermore, one sample for each of all the possible genotypes had formerly been confirmed by sequencing and severed as standards in the restriction analysis. For sequencing, PCR products overlapping both mutational sites of the TLR-2 gene were amplified using the following primers: forward 5′-CCCAGGAAAGCTCCCAGCAG-3′ and reverse 5′-GGAACCTAGGACTTTATCGCAGCTC-3′. PCR was performed in a total volume of 50 μl, and the final concentration of $MgCl_2$ was 1·5 mmol/l, the other components and concentrations being described as above. The PCR comprised an initial denaturation step of 10 min at 95°C, 35 cycles of denaturation for 30 s at 94°C, annealing for 30 s at 65°C and extension for 60 s at 72°C, and a final extension step for 10 min at 72°C. Before sequencing, the PCR products were purified using a Millipore Montage PCR purification kit (Millipore, Bedford, MA, USA). Dye terminator chemistry was used in these reactions, and sequences were resolved using the ABI 310 Genetic Analyser system. For sequence evaluation, the ABI Prism 230 DNA sequencing analysis software was used.

Statistical analysis

Demographic and clinical data are expressed as means±SD or percentage, and the overall genotype distributions and allele frequency at the 753 codon were compared between

Table 1 Clinical characteristics of patients (n=61)

	n	%
Sex		
Female	30	49.2
Male	31	50.8
Clinical symptoms and signs		
Arthritis	21	34.4
Carditis	18	29.5
Arthritis and carditis	17	27.9
Chorea	2	3.3
Carditis and chorea	3	4.29

patients and controls. The data were analysed as a 2×2 contingency table for carriage of allele *Gln*, as is the standard procedure. Odds ratios (ORs) with 95% confidence intervals (95% CI) were also calculated for these comparisons. Independent sample t test was used to assess the difference in several disease severity markers between TLR-2 *Arg753Arg* or *Arg753Gln* genotypes. Significance levels were set at 5% ($p<0.05$). Analyses were performed using the GraphPad Prism programme (version 4.0 for Windows, GraphPad Software, San Diego, CA, USA).

Results

Clinical features

The study consisted of 61 Caucasian Turkish ARF patients (31 male and 30 female; ages 11.16±2.88). Clinical characteristics of patients are presented in Table 1. The most frequent clinical symptoms were arthritis (34.4%), carditis (29.5%) and these two symptoms accompanying each other (27.9%). Serum CRP and ASO titers were determined during admission to the clinic with a rheumatic fever prediagnosis. Serum ASO and CRP were increased and in range typical of patients with ARF. Moreover, ESR and WBC counts were also significantly high in patients during diagnosis (Table 2).

We analysed the relationship between carriership of TLR-2 *Arg753Arg* or *Arg753Gln* genotypes and laboratory parameters. Although there is an insignificant tendency that TLR-2 *Arg753Gln* carriers in patients had different baseline laboratory characteristics, significant differences were only observed for ASO (p=0.037).

TLR-2 genotype distribution and allele frequency

Using blood samples encompassing Turkish ARF patients and child and adult controls, we tested for the association of predisposition to the disease with *Arg753Gln* and *Arg677Trp* polymorphisms in the TLR-2 gene. The findings are summarised in Tables 3 and 4. Results have shown that the *Arg753Arg* genotype was significantly decreased in the entire group of ARF cases when compared with healthy children controls (OR 0.0098, 95% CI 0.00312–0.00308, $p<0.0001$). ARF patients were also more frequently *Arg753Gln* heterozygote (OR 100, 95% CI 32–320, $p<0.0001$), and *Gln* allele distribution was different from that among control subjects (45.9% vs 4.95%, OR 16, 95% CI 7.6–35, $p<0.0001$) (Table 3).

To exclude persons who might have a genetic risk for ARF in childhood, we also evaluated the results according to an adult control group (Table 4). The *Arg753Arg* genotype was also significantly decreased in the entire group of ARF cases when compared with healthy adult controls (OR 0.0103, 95% CI 0.0034–0.0.031, $p<0.0001$). ARF patients were more frequently *Arg753Gln* heterozygote (OR 97.1, 95% CI 32.5–290, $p<0.0001$), and *Gln* allele distribution was different from that of control subjects (45.9 vs 5.2%, OR 15.6, 95% CI 7.87–30.8, $p<0.0001$). In contrast to the results for *Arg753Gln* polymorphism, we did not detect any person with *Arg677Trp* polymorphism in either the control or the patient group.

Discussion

The epidemiological association between group A β-hemolytic streptococcal infections and the subsequent development of ARF has been well established. ARF is a delayed autoimmune response to group A streptococcal pharyngitis, and the clinical manifestation of the response and its severity in an individual is determined by host genetic susceptibility, the virulence of the infecting organism and a conducive environment [7, 34, 35].

Although substantial progress has been made in the understanding of ARF as an autoimmune disease, the

Table 2 Baseline laboratory characteristics in ARF patients according to their genotype and normal serum reference of healthy children

Parameter (normal range)	ARF patients		
	Total group±SD (minimum–maximum)	*Arg753Arg*±SD (minimum–maximum)	*Arg753Gln*±SD (minimum–maximum)
ASO titer (<333 for children)	1,499.9±2,043.9 (200–15,000)	821±416 (441–1,440)	1,560.52±2,121.1[a] (200–15,000)
CRP (7–820) (μg/dl)	11.44±10.10 (0.86–70.4)	10.2±4.66 (4–16.3)	11.55±10.46 (0.86–70.4)
ESR (3–13 before puberty) (mm/h)	94.97±115.07 (12–960)	89.8±9.12 (75–98)	95.43±120.15 (12–960)
WBC cells (4.5–11.0) (×10^3 μl)	15,764±5,597 (4,800–34,000)	16,820±10,074 (9,900–34,000)	15,670±5,165 (4,800–28,000)

ASO Antistreptolysin O, *CRP* C-reactive protein, *ESR* erythrocyte sedimentation rate, *WBC* white blood cells, *M* male, *F* female
[a] *Arg753Gln* carriers in patients had significantly higher ASO titrations than *Arg753Arg* carriers (p=0.037)

Table 3 Genotype distribution and allele frequency at the TLR-753 locus in healthy children and in patients with ARF

TLR-2 *Arg753Gln* polymorphism	ARF patients (*n*=61)		Healthy child controls (*n*=91)		*p*	OR	95% CI
	Ages (mean±SD) 11.16±2.88		Ages (mean±SD) 8.71±1.3				
	n	%	*n*	%			
Genotype distribution							
Arg753Arg	5	8.2	82	90.11	<0.0001	0.0098	0.00312–0.0308
Arg753Gln	56	91.8	9	9.89	<0.0001	100	32–320
Gln753Gln	0	–	0	–	–		
Allele frequency							
Arg	66	54.1	173	95.05	<0.0001	16	7.6–35
Gln	56	45.9	9	4.95			

precise pathogenetic mechanism of ARF has not been defined. Major histocompatibility antigens, potential tissue-specific antigens and antibodies developed during and immediately after a streptococcal infection are being investigated as potential risk factors in the pathogenesis of the disease. Recent evidence suggests that T cell lymphocytes play an important role in the pathogenesis of RHD. It has also been postulated that particular M types of group A streptococci have rheumatogenic potential. However, encapsulation is not exclusive to these strains, and much of the data supporting the idea of selective "rheumatogenicity" is unreliable [34, 36].

Host–pathogen interaction in infection by streptococci begins with the binding of bacterial surface ligands to specific receptors on host cells and subsequently involves specific processes of adherence, colonization and invasion. The binding of bacterial surface ligands to host surface receptors is the most crucial event in the colonization of the host, and it is initiated by fibronectin and by streptococcal fibronectin-binding proteins [37]. Streptococcal lipoteichoic acid and M protein also play major roles in bacterial adherence [38]. The host responses to a streptococcal infection include type-specific antibody production, opsonization and phagocytosis. Initial streptococcal infection in a genetically predisposed host in a susceptible environment leads to the activation of T cell and B cell lymphocytes by streptococcal antigens and superantigens, which results in the production of cytokines and antibodies directed against streptococcal carbohydrate and myosin [37, 38].

Genetically programmed determinants of host susceptibility to ARF have been studied extensively in an attempt to determine why only 0.3–3% of individuals with acute streptococcal pharyngitis go on to develop rheumatic fever [34, 35]. Our findings related to the pharyngitis history of patients were consistent with the literature. Pedigree studies suggested that this immune response is genetically controlled, with high responsiveness to the streptococcal cell-wall antigen being expressed through a single recessive gene and low responsiveness through a single dominant gene. TLRs are key players in host immunity, and many examples suggest that pathogens can manipulate or evade the activation of TLRs. Experimental assessment of the specific role of this evasion in pathogenicity is often challenging. TLRs can initiate the entire host defence, be it innate or adaptive. TLRs are particularly notable in that they activate signalling pathways that culminate in the induction of all our favourite immune and inflammatory genes, including the major histocompatibility, co-stimulatory molecules, antibodies, cytokines, chemokines and adhesion molecules [39].

Recent studies have identified that TLR-2 interacts with a large number of agonists, including peptidoglycan and lipoteichoic acid of gram-positive, spirochetal glycolipids, lipoproteins and lipopeptides of *Borrelia* and *Mycoplasma* species [40–43]. Each TLR is a single-pass transmembrane receptor with an extracellular domain containing multiple leucine-rich repeats and an intracellular signalling domain that is homologous to the cytoplasmic tail of the interleukin

Table 4 Genotype distribution and allele frequency at the TLR-753 locus in healthy adult volunteers and in patients with ARF

TLR-2 *Arg753Gln* polymorphism	ARF patients (*n*=61)		Healthy adult controls (*n*=116)		*p*	OR	95% CI
	Ages (mean±SD) 11.16±2.88		Ages (mean±SD) 28.04±6.6				
	n	%	*n*	%			
Genotype distribution							
Arg753Arg	5	8.2	104	89.66	<0.0001	0.0103	0.0034–0.031
Arg753Gln	56	91.8	12	10.34	<0.0001	97.1	32.5–290
Gln753Gln	0	–	0	–	–		
Allele frequency							
Arg	66	54.1	220	94.8	<0.0001	15.6	7.87–30.8
Gln	56	45.9	12	5.2			

(IL)-1 receptor. Stimulation of TLRs direct the activation of nuclear transcription factor kappa B (NF-κB) and the production of pro-inflammatory cytokines [22].

It is also important to emphasise that TLRs can also induce responses that are not dependent on gene expression, such as in the promotion of phagocytosis, broadening their roles in host defence [44]. A lack of TLR-2 or its adaptors may severely immunocompromise the host [44]. This is particularly interesting because a dysregulation in TLR-2, either through under- or overactivation, is likely to lie at the heart of many pathologies, from sepsis to autoimmunity. In this study, we identified 56 children among 61 patients who were heterozygous for *Arg753Gln*, while no homozygous patients were found. However, nine child controls were heterozygous out of 91 healthy children, and 12 adult controls were heterozygous out of 116 adult volunteers. These results indicate that there is a strong significant relationship between ARF tendency and *Arg753Gln* polymorphism. Our result is the first in the literature to find a 97.1-fold tendency in a single nucleotide polymorphism's genotype and a 15.6-fold tendency in a specific allele of a gene for ARF. The results were more impressive when the child control group was considered to calculate ORs. An almost 100-fold tendency in the TLR-2 *Arg753Gln* genotype and a 16-fold tendency in the *Gln* allele of TLR-2 were determined. Moreover, we also found a positive relationship with the *Arg753Gln* genotype and serum ASO. Beside all related host and microbial molecules, TLR-2 may be a key player in ARF pathophysiology. Our data also indicate that *Arg753Gln* polymorphism occurs in 10.34% of Turkish people (9.89% in the child population), which is slightly higher than a German group has previously reported (9.4%) [33]. Meanwhile, no individuals carrying the *Arg677Trp* polymorphism were identified in the control and patient groups; this has previously been reported among Asians but is not reported among Europeans [33, 45]. Taken together with these data, our results can be regarded as representative.

Acute rheumatic fever is still an important health problem worldwide. Strong relations with socio-economical status and lack of treatment are major concerns especially in developing regions of the world. Thus, identifying a strong genetic factor might be very helpful to children under a high genetic risk for ARF. We found that polymorphism of the TLR-2 gene leading to a replacement of arginine by glutamine at position 753 leads to increased susceptibility to ARF. Experimental evaluation of the specific role of this evasion in pathogenicity is challenging. In addition to usage of TLR-2 as a genetic marker for risk stratification, it is possible that TLR-2 activation may be therapeutically manipulated for the prevention of ARF in the future. This might enable scientists to develop TLR-2 inhibitors, either through the use of soluble forms of TLR-2, neutralizing antibodies or inhibitors of intracellular signalling. The devastating effects of ARF will continue to perplex clinicians until such questions are answered.

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