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Cutting Edge

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Cutting Edge: Genetic Association between IFI16 Single Nucleotide Polymorphisms and Resistance to Genital Herpes Correlates with IFI16 Expression Levels and HSV-2–Induced IFN-β Expression

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IFN-γ–inducible protein 16 (IFI16) is an immunological DNA sensor proposed to act in the cyclic GMP–AMP synthase–stimulator of IFN genes pathway. Because mice do not have a clear ortholog of IFI16, this system is not suitable for genetic studies of IFI16. In this study, we have compared the dependency on IFI16, cyclic GMP–AMP synthase, and stimulator of IFN genes for type I IFN induction by a panel of pathogenic bacteria and DNA viruses. The IFN response induced by HSV-2 was particularly dependent on IFI16. In a cohort of patients with genital herpes and healthy controls, the minor G allele of the *IFI16* single nucleotide polymorphism rs2276404 was associated with resistance to infection. Furthermore, the combination of this allele with the C allele of rs1417806 was significantly overrepresented in uninfected individuals. Cells from individuals with the protective GC haplotype expressed higher levels of IFI16 and induced more IFN-β upon HSV-2 infection. These data provide genetic evidence for a role for IFI16 in protection against genital herpes. ***The Journal of Immunology*****, 2017, 199: 2613–2617.**

The presence of DNA in the cytoplasm represents a danger signal and is detected by pattern recognition receptors to evoke innate immune responses (1). Two key pathways stimulated by DNA are the inflammasome and type I IFN pathways. Whereas absent in melanoma 2 (AIM2) is the DNA sensor triggering inflammasome activation and downstream IL-1β maturation and pyroptosis, cyclic GMP–AMP (cGAMP) synthase (cGAS) is the essential DNA sensor mediating production of type I IFN, which has potent antiviral activity (2, 3). Following DNA recognition, cGAS produces 2′3′-cGAMP, which acts as a second messenger and binds to stimulator of IFN genes (STING), thus activating a signaling pathway involving the kinase TANK-binding protein 1 (TBK1) and the IFN-inducing transcription factor IFN regulatory factor 3 (2, 4).

In addition to cGAS, several other DNA sensors have been proposed, including IFN-γ–inducible protein 16 (IFI16) (1, 5). IFI16 is a member of the PYHIN protein family, which also includes AIM2, and this family of proteins has been named AIM2-like receptors (ALRs). Recently, mice lacking the whole ALR locus were generated and found not to have defects in the DNA-stimulated IFN induction pathway (6). However, mice do not have a clear ortholog of IFI16, and the murine system is therefore not suitable for genetic studies of IFI16. Recently, three studies have reported that IFI16 acts in the cGAS–STING pathway by promoting DNA sensing as well as cGAMP-mediated recruitment of TBK1 to STING (7–9). However, no human genetic data have been published to demonstrate a role for IFI16 in defense against DNA pathogens. In this study, we wanted to characterize the role of IFI16 in the innate response to human pathogenic viruses and bacteria, and also to explore whether genetic data from patient cohorts would support a role for IFI16 in host defense.

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Abbreviations used in this article: AIM2, absent in melanoma 2; ALR, AIM2-like receptor; cGAMP, cyclic GMP–AMP; cGAS, cGAMP synthase; IFI16, IFN-γ–inducible protein 16; MOI, multiplicity of infection; SNP, single nucleotide polymorphism; STING, stimulator of IFN genes; TBK1, TANK-binding protein 1.

Materials and Methods

Cells

THP1 cells (American Type Culture Collection) were cultured as nonadherent monocyte-like cells in normal growth media (RPMI 1640 containing 10% [v/v] FCS, 600 μg of L-glutamine per milliliter, 200 IU of penicillin per milliliter, and 100 μg of streptomycin per milliliter). Genome-edited THP1-derived cells lacking cGAS, IFI16, or STING were generated as described previously (7, 10). THP1 cells were differentiated into macrophage-like cells by addition of 150 nM PMA (Sigma-Aldrich). All experiments with THP1 cells were performed on differentiated THP1. For stimulation experiments, the cells were seeded in 48-well plates at a density of 50,000 cells per well. The cells were infected with the viruses and bacteria as specified in the figure legend and incubated for 18 h prior to isolation of supernatants. Frozen PBMCs were thawed in 50-ml tubes in 20 ml of preheated growth media and spun down at 350 × *g* for 10 min. PBMCs were distributed into 24-well plates at a concentration of 10^6 cells per 300 μl of media. Cells were incubated overnight at 37°C before further treatment.

Viruses and bacteria

The following viruses and bacteria were used for infection experiments: HSV-1 (strain KOS), HSV-2 (strain 333), HCMV (strain AD169), *Listeria monocytogenes* (strain LO28), and *Neisseria meningitidis* (strain NGO93). The microorganisms were propagated as described previously (11–14). For HSV-2, briefly, supernatants were harvested 2–3 d after infection of Vero cells at a multiplicity of infection (MOI) of 0.01. The virus was retrieved by one cycle of freeze-thawing followed by centrifugation to remove cellular debris. Four HSV-2 isolates were collected from patients with genital lesions visiting a sexually transmitted disease clinic in Gothenburg, Sweden (B4327UR, 90036, and 90263) and in Dar es Salaam, Tanzania (T-25/2557). The B4327UR has been used as a laboratory strain for several years in Gothenburg and has been passaged 10 times. The other isolates have a low passage number (≤4). The isolates were typed by an HSV-2–specific PCR method (15). The US4, US7, and US8 regions of the strains B4327UR and T-25/2557 have been sequenced earlier (16). The behavior in cell culture has previously been reported for the strains 90036 and 90263 (17).

Measurement of type I IFN bioactivity

Bioactive type I IFN was measured on cell supernatants by use of HEK-Blue IFN-α/β cells as reporter cells according to the manufacturer's instructions (InvivoGen). The HEK-Blue IFN-α/β cell line is generated from HEK293 cells stably transfected to express STAT2 and IFN regulatory factor 9 as well as a reporter gene construct with the gene encoding secreted embryonic alkaline phosphatase under the control of ISG54 promoter. Secreted embryonic alkaline phosphatase activity was quantified using QUANTI-Blue (InvivoGen). The QUANTI-Blue color change was measured at 620 nm on an ELx808 (BioTek). For the generation of standard curves either human IFN-α or human IFN-β (both PBL Assay Science) were used.

RT-PCR

IFN-β gene expression was determined by real-time PCR using TaqMan detection systems (Applied Biosystems). Expression levels were normalized to β-actin expression. The primer probes used were Applied Biosystems TaqMan assays: IFN-β, Hs01077958_s1; Mx1, Hs00895608; β-actin, Hs99999903_m1; and IFI16, Hs00986757_m1.

Human sample collection

The genetic material consisted of genomic DNA from 227 HSV-2–infected individuals and 232 HSV-2–seronegative healthy controls. The group of HSV-2–infected individuals consisted of 56% males and 44% females recruited from the sexually transmitted infections clinics at the Sahlgrenska University Hospital, the Borås Hospital, and the Uddevalla Hospital in Sweden. The average age was 39 y for men (range, 20–70 y) and 37 y for women (range, 20–68 y). HSV-2 infection was confirmed serologically using an HSV-2 ELISA kit (HerpesSelect2 ELISA IgG; Focus Technologies). Coinfection with HSV-1 was assessed using an HSV-1 ELISA kit (HerpesSelect1 ELISA IgG; Focus Technologies). Of the group, 52% (n = 118) were seropositive for HSV-1, 45% (n = 102) were seronegative for HSV-1, and data were missing for 3% (n = 7).

Three individuals were on immunosuppressive treatment (against rheumatoid arthritis, ankylosing spondylitis, and CNS vasculitis) at the time of sampling. Other diseases that were present among the study population were multiple sclerosis (one patient), lichen sclerosis (one patient), and untreated ankylosing spondylitis and psoriasis (one patient).

Control individuals. For the control group, 232 age- and gender-matched healthy HSV-2–seronegative adult blood donors were recruited from the Blood Bank at Sahlgrenska University Hospital or from the Epidemiological Investigation of Rheumatoid Arthritis study controls (18). This anonymous group consisted of 58% males and 42% females with an average age of 47 and 38 y, respectively (range, 20–65 and 20–69 y, respectively). Of the control group, 69% (n = 161) of the individuals were seropositive for HSV-1, 24% (n = 56) were seronegative for HSV-1, and HSV-1 data were missing for 6% (n = 15). All individuals were screened for HSV-2 infection with ELISA. All individuals were routinely screened (and found to be negative) for blood-derived contaminating diseases, including hepatitis A, B, and C, HIV-1 and -2, and human T lymphotropic virus-1 and -2. Permission for this study was granted by the Ethics Committee of the University of Gothenburg, and all patients gave written informed consent.

Genotyping

DNA used for genotyping was extracted from heparinized or EDTA venous blood using the salting-out method or an E.Z.N.A blood DNA kit (Omega Biotec, Norcross, GA). Genotyping was performed for 227 HSV-2–infected individuals and 232 HSV-2–seronegative control subjects for five single nucleotide polymorphisms (SNPs) in *IFI16* (Supplemental Table I) using TaqMan allelic discrimination (premade or customized Applera assays) at the Core Facility at the Sahlgrenska Academy, Gothenburg University, Sweden. The SNPs were chosen based on being reasonably common among the European white population (minor allelic frequency of >0.10, according to the National Human Genome Research Institute's haplotype map; available at: http://www.genome.gov/Pages/Research/HapMap) and being spread out over the whole gene. The genotyping rate was 79% or higher (range, 79–98%).

ELISAs

For detection of HSV-specific Abs, plasma samples were screened for anti–HSV-2 glycoprotein G using HerpesSelect2 ELISA IgG (Focus Technologies) and for HSV-1 antibodies using HerpesSelect1 ELISA IgG (Focus Technologies). IL-6 was detected using the DuoSet ELISA kit from R&D Systems.

Statistical analyses

Genotype and allele frequencies were compared using a Fisher exact test and χ^2 test, respectively. The calculations were made with an on-line program (http://www.quantitativeskills.com/sisa/index.htm) or SPSS. A Hardy–Weinberg test, haplotype association test, and permutation test were performed using Haploview software. All allele and genotype frequencies were found to be in Hardy–Weinberg equilibrium. Statistical analyses of in vitro data were performed using a Student t test. Differences were considered significant at $p < 0.05$.

Results and Discussion

IFI16 is essential for stimulation of type I IFN expression by HSV-2

IFI16 is predominantly a nuclear protein, but it also exhibits cytoplasmic localization in selected cell types, such as macrophages (19), and can be induced to translocate to the cytoplasm in other cell types, such as keratinocytes (20).

In a first series of experiments we were interested in examining the IFI16 dependency for IFN-β induction by a series of viruses and bacteria. Control, $cGAS^{-/-}$, $IFI16^{-/-}$, or $STING^{-/-}$ PMA-differentiated THP1 cells, which exhibit a macrophage-like phenotype (13), were treated with the pathogens or synthetic nucleic acids. No overt differences in viability of cell lines receiving a given treatment were observed (data not shown). Supernatants and total RNA was isolated for measurement of type I IFN bioactivity and mRNA encoding the IFN-stimulated gene Mx1, respectively (Fig. 1A–1G, Supplemental Fig. 1A). Interestingly, whereas all microbes stimulated type I IFN production in a manner dependent on cGAS and STING, they differed substantially in the extent to which this response was dependent on IFI16 (Fig. 1H). The observed effects of IFI16 deficiency was not due to an effect on inflammasome activation, because treatment with the pan-caspase inhibitor Z-VAD-FMK (100 μM) did not significantly alter the induction of type I IFN by the stimuli used (data not

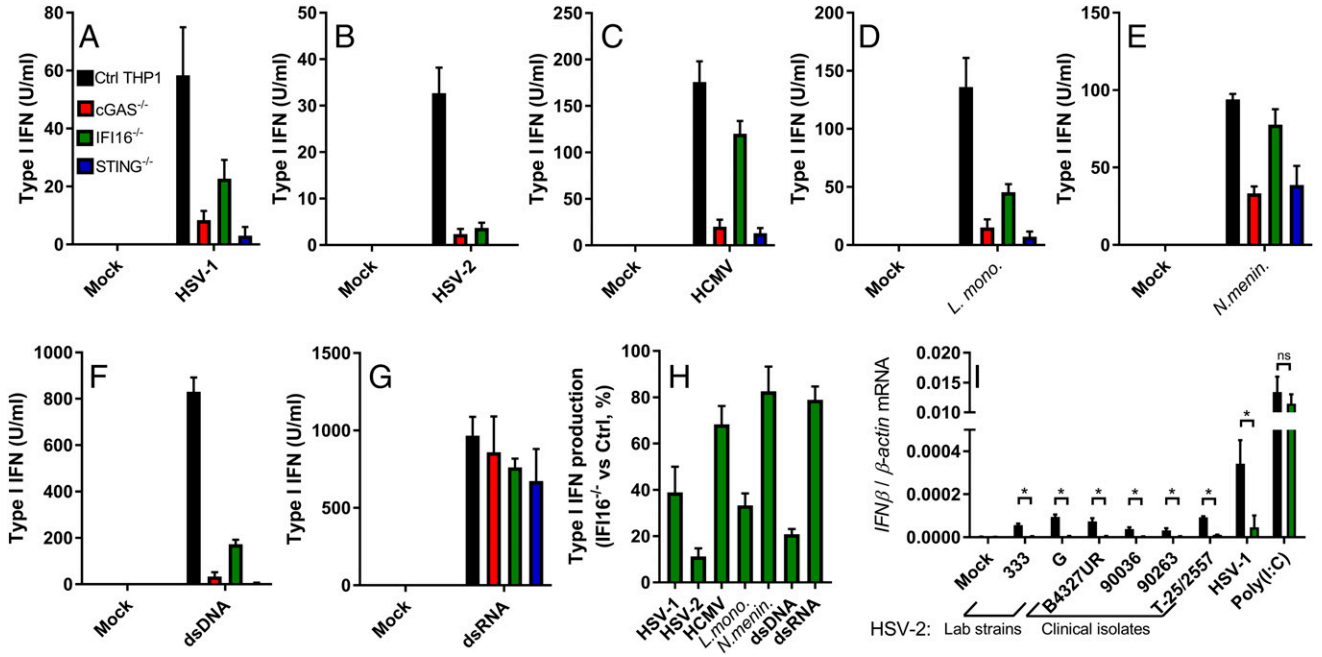

FIGURE 1. Role for IFI16 in stimulation of type IFN production by viruses and bacteria. The indicated THP1-derived cells were PMA differentiated and treated with the following microorganisms: (**A**) HSV-1 (MOI of 3), (**B**) HSV-2 (MOI of 3), (**C**) HCMV (MOI of 1), (**D**) *L. monocytogenes* (MOI of 25), (**E**) *N. meningitidis* (MOI of 10), (**F**) dsDNA (2 μg/ml), (**G**) dsRNA (2 μg/ml). Supernatants were isolated 16–18 h later and levels of type I IFN bioactivity were measured. (**H**) Relative levels of type I IFN production by IFI16$^{-/-}$ cells compared with control THP1 cells receiving the same stimulus. (**I**) Control and IFI16$^{-/-}$ THP1-derived cells were PMA differentiated and treated with the indicated laboratory-adapted HSV-2 strains or clinical isolates (MOI of 3), HSV-1 (MOI 3), or polyinosinic-polycytidylic acid [poly(I:C); 2 μg/ml]. Supernatants were isolated 16–18 h later and levels of type I IFN bioactivity were measured. All data are shown as mean of triplicates ± SD. The results shown are representative of three to four independent experiments. *$p < 0.05$. ns, not significant ($p > 0.05$).

shown). Importantly, HSV-2–induced type I IFN production was particularly dependent on IFI16. The highly IFI16-dependent nature of the induction by HSV-2 was seen for both laboratory-adapted HSV-2 strains and clinical isolates (Fig. 1I). When measuring the inflammatory cytokine IL-6, we observed a pattern similar to the type I IFN measurements for the virus-infected cells, whereas this response was totally independent of cGAS, IFI16, and STING in bacteria-infected cells (Supplemental Fig. 1B). Thus, among the microbes tested, IFI16 was particularly important for induction of type I IFN production in response to HSV-2.

The G allele of the IFI16 SNP rs2276404 is associated with natural resistance to genital HSV-2 infection

Given the identified role for IFI16 in induction of type I IFN expression by HSV-2 in cell culture, we were interested in determining whether genetic data would support a role for this DNA sensor in host defense. To this end, we examined five *IFI16* SNPs for association with susceptibility or resistance to genital HSV-2 infection. These were rs9887904, rs1417806, rs2276404, rs1772408, and rs866484. DNA was isolated from 227 HSV-2–infected individuals and 232 HSV-2–seronegative healthy controls. Interestingly, the minor G allele at rs2276404, situated in the 5′ untranslated region of the *IFI16* mRNA, was correlated with natural resistance to genital HSV-2 infection, as it was significantly more common among HSV-2–seronegative individuals compared with HSV-2–infected individuals (Table I). The G allele was found in 17% of the HSV-2–uninfected individuals compared with 12% of the HSV-2–infected individuals ($p = 0.0286$). We also assessed the genotype frequencies at the rs2276404 in HSV-2–negative controls and HSV-2–infected individuals. The homozygous GG genotype was found solely among uninfected control subjects and not in HSV-2–infected individuals ($p = 0.0249$) (Supplemental Table I). The GG genotypes were found in 3% of the control subjects, whereas 28% of the individuals in this group and 23% of the HSV-2–infected individuals carried the heterozygous AG genotype (Supplemental Table I).

To further test the role of genetic variations in HSV-2 infection, we analyzed different haplotype combinations in the *IFI16* gene containing the rs2276404 SNP. We found that the G allele at rs2276404 in combination with the C allele at rs1417806 were significantly more common in HSV-2–uninfected individuals compared with HSV-2–infected individuals (9.5

Table I. Allele frequencies of five IFI16 SNPs in HSV-2–infected and HSV-2–seronegative individuals

IFI16 SNP	Minor Allele	Allele Frequency (%)		p Value (χ^2)
		Uninfected	Infected	
rs9887904	A	11 (48/438)	9 (41/444)	0.462
rs1417806	C	31 (141/456)	26 (115/438)	0.1229
rs2276404	G	17 (76/450)	12 (48/412)	0.0286
rs1772408	A	21 (88/416)	16 (49/314)	0.0573
rs866484	C	25 (113/454)	25 (109/436)	0.907

Table II. Haplotype frequencies in Swedish HSV-2–infected and HSV-2–seronegative individuals for the rs22776404 and rs1417806 SNPs in the IFI16 gene

Haplotype[a]	Frequency (%)		p Value (χ^2)
	Uninfected	Infected	
AA	62	65	0.3032
CA	22	23	0.6876
CG	9.5	5.2	0.0185 (0.0402)[b]
AG	7.5	7.2	0.8678

[a]Estimated by Haploview 4.2 in order: rs1417806, rs22776404.
[b]p Value after 100,000 permutation tests.

and 5%, respectively) ($p = 0.0103$) (Table II). This difference remained significant also after 100,000 permutation tests ($p = 0.0202$), which was not the case for the results described above.

IFI16 expression levels in the haplotypes correlates with IFN expression and protection against genital herpes

When the *IFI16* mRNA levels were compared in patients from the protective CG haplotype group and patients from the susceptible AA haplotype group, we found higher *IFI16* mRNA levels in PBMCs from the CG haplotype group (Fig. 2A), but no overt difference in cell viability. Interestingly, following HSV-2 infection of PBMCs with the AA or CG haplotype, only the cells with the CG halpotype responded with detectable production of IFN-β (Fig. 2B). Moreover, cells with the CG haplotype also produced higher levels of IFN-β mRNA in response to dsDNA transfection than did cells with the AA haplotype, whereas the cells with the two haplotypes produced comparable levels of IFN-β after transfection of the dsRNA mimic polyinosinic-polycytidylic acid (Fig. 2C). We genotyped THP1 cells and found that they had the AA haplotype (data not shown). Interestingly, similar to PBMCs with the AA haplotype, undifferentiated THP1 cells express very low levels of IFI16 and have low responsiveness to foreign DNA (13). Thus, the *IFI16* rs2276404 is associated with natural resistance to genital HSV-2 infection, which correlates with expression of IFI16 and HSV-2–induced IFN-β expression.

IFI16 was identified as an innate DNA sensor involved in DNA-induced type I IFN expression (5). Subsequent work has confirmed the important role for IFI16 in DNA-stimulated IFN responses (21–23). However, some studies have not been able to find a role for IFI16 in immunological DNA sensing (2, 24, 25), maybe suggesting a specialized or cell type–specific role for IFI16 in this process. Mice deficient in all ALRs have been reported to have normal IFN response to foreign DNA (6). However, because mice do not have a clear ortholog of IFI16, the murine system does not seem to be well suited to study the role of IFI16 in DNA-stimulated IFN responses. Recently, it was reported from independent groups that IFI16 augments DNA-stimulated cGAS-mediated cGAMP production and type I IFN expression (7, 9), and also supports recruitment of TBK1 to STING (7, 8). This suggests that IFI16 is indeed a DNA sensor, either directly or as a cofactor for cGAS.

In this study we have evaluated the role of IFI16 in stimulation of type I IFN production in a human macrophage-like cell line in response to a range of human pathogens. We found that although all pathogens stimulated the response in a cGAS-STING–dependent manner, there was a large variation in the dependency on IFI16. These data suggest either that IFI16 is not an essential part of the cGAS–STING pathway, but rather an amplifying cofactor, or alternatively that the microbes tested also stimulate other signaling pathways, which could compensate for IFI16 in IFN induction. In light of the conflicting literature on IFI16 and DNA-stimulated IFN expression, much of which is based on work with synthetic DNA, the authors favor the former explanation.

The in vitro analysis revealed that the type I IFN expression stimulated by HSV-2 was particularly dependent on IFI16. The subsequent genetic analysis of IFI16 SNPs in females with genital herpes led to identification of SNPs that are associated with resistance to disease. Moreover, analysis of combinations of haplotype frequencies revealed that the combination of the

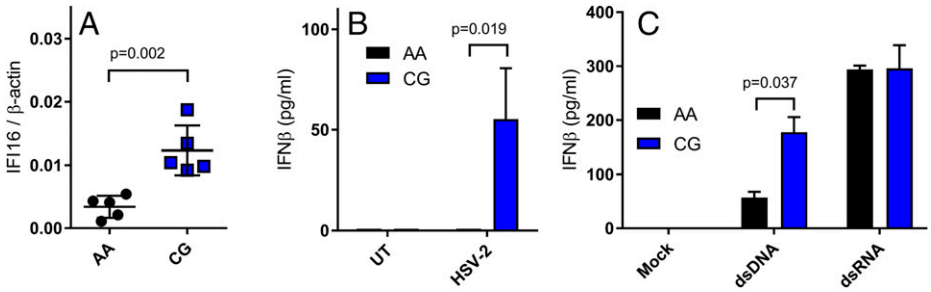

FIGURE 2. Characterization of PBMCs with the AA and CG haplotypes in the rs22776404 and rs1417806 SNPs in the IFI16 gene. (**A**) Total RNA was isolated from individuals with the AA and CG haplotypes in the rs22776404 and rs1417806 SNPs in the IFI16 gene, and mRNA levels of IFI16 and β-actin were measured by real-time quantitative PCR. Data are shown as individual data points with the mean represented with a line. (**B** and **C**) PBMCs from individuals with the AA and CG haplotypes in the rs22776404 and rs1417806 SNPs in the IFI16 gene were (B) infected with HSV-2 (MOI of 3) or (C) stimulated with dsDNA (2 μg/ml) or dsRNA (2 μg/ml). Supernatants were isolated 18 h later and levels of IFN-β were measured by ELISA. The data are shown as mean ± SD. All data shown included five donors per group.

G allele of rs2276404 and the C allele of rs1417806 was significantly more common in HSV-2–uninfected individuals, which correlated with higher IFI16 expression and IFN induction after HSV-2 infection in vitro.

Collectively, we demonstrate that induction of type I IFN expression by HSV-2 in human cells is highly dependent on IFI16 and provide the genetic evidence that links IFI16 haplotypes with susceptibility to genital herpes. Importantly, cells with the protective IFI16 haplotype have higher IFI16 expression and induce higher IFN-β expression after HSV-2 infection. To our knowledge, this study thus provides the first genetic evidence for a protective role for IFI16 in host defense in human immunology.

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Disclosures

The authors have no financial conflicts of interest.

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