Molecular profiling of primary uveal melanomas with tumor-infiltrating lymphocytes

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Abbreviations: CCL20, macrophage inflammatory protein-3a/liver and activation-regulated chemokine; CCL4, macrophage inflammatory protein-1ß; CCL5, RANTES; CCR, CC receptor; CD3D, CD3d, molecule, delta; CD3E, CD3e molecule, epsilon; CD3G, CD3γ molecule, gamma; CD3Z, T cell receptor zeta chain; CTLA4, cytotoxic T-lymphocyte antigen 4; CXCR, (C-X-C) motif chemokine receptor; FASLG, fas ligand; GATA3, GATA Binding Protein 3; GZMB, granzyme; IFNG, interferon γ; INDO, indolamine dehydrogenase; LAG3, lymphocyte-activation gene 3; MHC, major histocompatibility; NK, natural killer; PD-L1, programmed cell death-1 ligand; PDCD1, programmed cell death-1; PRF1, perforin; PTCRA, pre T-cell antigen receptor α; RORC, RAR-Related Orphan Receptor C; TBX21, T-Box 21; TCR, T cell receptor; TGF, transforming growth factor; TILs, tumor-infiltrating lymphocytes; Treg, regulatory T cell

In contrast to other cancers, the presence of tumor-infiltrating lymphocytes (TILs) in uveal melanoma is associated with a poor prognosis. However, how TILs may promote disease progression and what regulates their infiltration has not yet been established. To address these clinically relevant outstanding questions, T cell, immune regulatory, and chemokine gene expression profiles of 57 enucleated uveal melanoma tumors were compared, encompassing 27 with TILs and 30 without. Tumors with infiltrating lymphocytes expressed more CD8A mRNA, as well as IFNG, TGFß1, and FOXP3 transcripts. Other T helper associated cytokines and T helper transcription factors were not differentially expressed, nor were mediators of lymphocyte cytotoxicity. The immune inhibitors INDO, PDCA1, CTLA4, and LAG3, and the non-classical MHC Class I target of CD8 T regulatory cells, HLA-E, were significantly higher in tumors with TILs. FAS was also significantly higher. The C-C chemokine ligands CCL4, CCL5, and CCL20 were higher in tumors with TILs. Levels of CCL5 were most strongly correlated with levels of CD8A. Chemokine receptors were not differentially expressed. Molecular profiling of uveal melanoma tumors with TILs supports the existence of an immunosuppressive tumor microenvironment and suggests roles for CD8 T regulatory T cells, as well as specific chemokines, in fostering uveal melanoma disease progression.

Introduction

Melanoma of the eye’s uveal tract is fatal in almost half of all patients afflicted with this malignancy due to the development of metastatic disease. Unique immunological mechanisms have been implicated in regulating the clinical course of uveal melanoma. In many solid tumors, including cutaneous melanoma, the presence of tumor-infiltrating lymphocytes (TILs) is associated with a better prognosis.¹⁻³ However, in uveal melanoma TILs correlate with the development of metastatic disease and consequently, a worse prognosis.⁴ Elevated tumor major histocompatibility (MHC) Class I antigen expression, which is necessary for T-cell recognition but which renders cancer cells resistant to natural killer (NK) cells, is also associated with the development of metastases in uveal melanoma.⁵,⁶ This too contrasts with cutaneous melanoma in which low expression of MHC Class I is associated with increased tumor thickness and a poorer prognosis.⁷

How TILs promote uveal melanoma progression has not yet been established. The eye is considered to be an “immune-privileged” site where both innate and adaptive immunity are suppressed by anatomical, physiological, and immunoregulatory mechanisms. TILs in uveal melanoma have been shown to be predominantly CD8+ T cells; the frequency of CD4+ T cells is low, and B cells and natural killer (NK) cells are only rarely identified.⁸⁻¹² Uveal melanoma cells have been shown to produce several immunosuppressive factors, including indolamine 2,3-dioxygenase 1 (IDO1, better known as INDO) and transforming growth factor β (TGFß).¹³,¹⁴ Reduced expression of the T cell receptor (TCR) zeta chain (CD247/CD3z), an indicator of T-cell suppression, has also been reported among uveal melanoma TILs.⁹ On the contrary, CD4+FoxP3+ regulatory T (Treg) cells that suppress antitumor immune responses by a variety of mechanisms are infrequent and do not appear to have independent prognostic significance.¹⁵,¹⁶
How lymphocyte infiltration into uveal melanoma lesions is regulated has also not been established. Melanoma cells have been reported to produce both C-C and C-X-C motif chemokines, central regulators of lymphocyte trafficking. In cutaneous melanoma, CCL2, CCL3, CCL4, CCL5, CXCL9, and CXCL10 have been found to be preferentially expressed in tumors that contained T cells. A variety of chemokine receptors are also purportedly expressed by melanoma cells. In cutaneous melanoma the C-X-C chemokine receptor 3 (CXCR3) has been associated with an absence of TILs and a poorer prognosis. Uveal melanoma cells have been shown to express CCL2, CXCL1, CXCL8, and the chemokine receptors CCR7, CXCR1, CXCR2, and CXCR4. There is, however, little definitively known about the relationship between TILs and chemokines and their receptors in uveal melanoma tumors.

Molecular profiling techniques have been previously applied to examine intratumoral immune responses in cutaneous melanoma, lymphoma, and several carcinomas, including breast, colorectal, and hepato-cellular. Here, we apply gene expression profiling to comparatively examine uveal melanoma tumors with, and without, TILs, focusing on T-cell, immune regulatory, and chemokine-associated transcripts.

**Results**

Uveal melanomas from 57 enucleations, 27 (47%) with TILs as defined as >100 lymphocytes in 20 high power fields, and 30 (53%) without, were analyzed for the levels of immune associated transcripts. As would be expected and as shown in Figure 1, tumors with TILs expressed significantly higher levels of subsets is displayed in Figure 3. Significant increases in the transcript levels of the Th1-associated interferon γ (IFNG) and the immunosuppressive Treg product TGFB1 characterized tumors with infiltrating lymphocytes. The Treg-associated IL10 was very highly but not differentially expressed. Expression of the Th2-associated IL4 and IL13 and Th17-associated cytokines was low. Significant increases in the transcript levels of the Treg-associated transcription factor, FOXP3, were also observed. That tumors harboring infiltrating lymphocytes expressed FOXP3 was confirmed by IHC (Fig. 2A). The Th1-associated transcription factor TBX21 was not differentially expressed, nor were the Th2-associated GATA3 or the Th17-associated RORC. Transcripts for mediators of lymphocyte cytotoxicity, including granzyme (GZMB), perforin (PRF1), and Fas ligand (FASLG), tended to be higher in tumors with TILs, but differences did not reach statistical significance (Fig. 4). Transcript of T cell intracellular antigen 1 (TIA1), an antigen associated with cytolytic cytotoxic granules of T cells, also was not differentially expressed. The death receptor FAS, an indicator of cell death, including lymphocyte death was more highly expressed with TILs than without.

Several negative immune regulators were more highly expressed in tumors with TILs (Fig. 5A) relative to TIL-negative patient samples. These included INDO as well as PDCA1 (programmed death-1; PD-1), CTLA4 (cytotoxic T-lymphocyte antigen 4), and LAG3 (lymphocyte-activation gene 3). The CTLA4 ligand CD86 was also higher in tumors with TILs whereas the PD-1 ligand PD-L1 and the CTLA4 ligand CD80 were not. Given that TIL-positive tumors were characterized by increases in CD8 and FOXP3 expression, as well as higher transcript levels for IFNG, INDO and LAG3, the possibility that CD8+ Treg cells were involved was examined by assessing non-classical MHC molecules recognized transcripts encoding T cell receptor (TCR) components, including pre TCR (PTCRA), CD3ζ (CD3D), CD3ε (CD3E), and CD3ζ (CD3G) expression was low. Tumors with TILs also expressed significantly more CD8α than CD4. In one of the 27 tumors with TILs, only CD8 transcripts were detectable and in 17 others, intensity of CD8α over CD4 was >2 fold. CD4 expression intensity was greater than CD8 in 9 tumors with TILs, >2 fold in 3. That the TILs were predominantly CD8+ and not CD4+ was confirmed by IHC (Fig. 2A). The expression of cytokines and transcription factors associated with T helper (Th)
by CD8+ Treg cells (Fig. 4B). The expression of HLA-E, but not HLA-F and HLA-G, was higher in tumors with lymphocytic infiltration versus those without (Fig. 5B).

The expression of various chemokines and chemokine receptors with and without TILs is summarized in Table 1. Most C-C and C-X-C motif chemokine transcripts assessed were present in all tumors with TILs. These included CCL2, CCL3, CCL4, CCL5, CXCL9, and CXCL10. Only CCL4 (macrophage inflammatory protein-1β) and CCL5 (RANTES), however, were higher in tumors with TILs compared to tumors without TILs. That TILs expressed CCL5 was confirmed by IHC (Fig. 2C). Although expression was lacking in approximately 20% of tumors with TILs, higher levels of CCL20 (macrophage inflammatory protein-3α/liver and activation-regulated chemokine) were also observed. Correlations between CD8 and all chemokines tested were evaluated. CCL5 expression was the most strongly correlated (Fig. 6). Transcripts of C-C motif and C-X-C motif chemokine receptors were not detected in most tumors, and differential expression was not apparent.

**Discussion**

In contrast to several other cancer types in which infiltrating lymphocytes are a good prognostic indicator, the presence of TILs in uveal melanoma confers a poor prognosis. Molecular profiling performed here supports the existence of an immunosuppressive, tumor-promoting microenvironment in uveal

![Figure 2. Lymphocytes infiltrating uveal melanomas express CD8, FOXP3 and CCL5.](image)

![Figure 3. Patient uveal melanomas with and without tumor-infiltrating lymphocytes express similar T helper cell gene expression profiles.](image)
melanoma tumors with infiltrating lymphocytes. Transcripts of the immunosuppressive molecules TGF\(\beta\) and INDO, potentially arising from a variety of cell types present in the tumor microenvironment, were increased in tumors with infiltrating lymphocytes. We also detected an increase in transcripts encoding CTLA-4, PD-1, and LAG-3, cell-intrinsic negative regulatory molecules that regulate T cells to promote tumor immune escape. All 3 have also been implicated in CD8\(^+\) T-cell exhaustion and in inhibiting the accumulation of self-reactive CD8\(^+\) T cells. IFN\(\gamma\) (IFNG) transcripts were also increased. While a central supporter of antitumor Th1-associated cellular immunity, not only can IFN\(\gamma\) promote non-classical MHC expression, it can also induce the expression of inhibitory molecules, including INDO, transcripts of which were also increased.\(^{27}\) In fact, IFN\(\gamma\) can inhibit immunotherapy by inducing apoptosis of CD4\(^+\) T cells.\(^{28}\) Furthermore, uveal melanoma cells treated with IFN\(\gamma\) resist lymphocytic granule-mediated target cell lysis.\(^{29}\) That the Th1-associated transcription factor, TBX21, was not differentially expressed would also be consistent with the lack of an antitumor Th1-associated response.\(^{30}\) HLA-E expression, which also characterized tumors infiltrated by lymphocytes, has been implicated in cancer cell escape from immunosurveillance as a consequence of its capacity to bind CD8\(^+\) Tregs as well as inhibitory NK cell receptors.\(^{31}\) The increase in HLA-E, which is expressed low levels by many tissues, but not HLA-G, another non-classical MHC target of CD8\(^+\) Treg cells, parallels independent studies of uveal melanoma cells lines in vitro in which HLA-E is expressed and further enhanced by IFN\(\gamma\) treatment, whereas HLA-G is neither expressed nor enhanced by IFN\(\gamma\).\(^{32}\)

Although CD4 expression was observed and did predominate in some tumors, TILs were predominantly CD8\(^+\) as supported by the gene expression profiling and confirmed by IHC, findings consistent with previous reports.\(^{8-12}\) The increases in FOXp3, IFNG, TGF\(\beta\)1, INDO, LAG3, and HLA-E suggest that tumor-infiltrating CD8\(^+\) cells may function as Tregs. An important contribution to immune privilege of the eye is a unique immune deviation that is elicited when antigens are introduced into

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**Figure 4.** Transcriptional analysis of mediators of lymphocyte cytotoxicity in uveal melanoma patient samples. Expression intensity of the transcripts of mediators of lymphocyte cytotoxicity of uveal melanomas with tumor-infiltrating lymphocytes (TIL; \(n = 27\)) compared to tumors without \((n = 30)\). RNA was extracted from tumor specimens and gene expression profiling performed using Illumina Sentrix 8 BeadChip arrays. Bars represent the mean ± SEM. Statistical analysis was performed using a 2-sided Student’s t tests after log transformation and the P-values were adjusted for multiple testing according to the methods of Benjamini and Hochberg; P-values were >0.05 for all comparisons.

**Figure 5.** T cell immune checkpoint molecules and negative regulators are differentially expressed in uveal melanoma tumor specimens. Expression intensity of the transcripts of (A) negative immune regulators, (B) their ligands, and (C) non-classical MHC Class I targets of tumors with tumor-infiltrating lymphocytes (TIL; \(n = 27\)) compared to tumors without \((n = 30)\). RNA was extracted from tumor specimens and gene expression profiling performed using Illumina Sentrix 8 BeadChip arrays. Bars represent the mean ± SEM. Statistical analysis was performed using a 2-sided Student’s t tests after log transformation and the P-values were adjusted for multiple testing according to the methods of Benjamini and Hochberg; brackets indicate statistically significant differences between the groups with the P-value indicated above.
Tregs that can inhibit both Th1- and Th2-associated immune responses. Further supporting this line of reasoning, melanoma-induced CCL5 production by infiltrating T cells has been shown to inhibit T-cell activation by interfering with T cell receptor signaling. The presence of CD8+ cells in uveal melanoma, in particular, has been associated with lower metastasis-free survival. Proinflammatory cytokines, such as TNFα and IFNγ, can promote Th1-associated antitumor responses. A recent clinical cancer immunotherapy trial has included pre-treatment tumor gene expression profiling, and clinical benefit has been shown to correlate with a gene signature that included T-cell and chemokine markers. High expression of CCL5, CXCL9, and CXCL10 were among those differentially expressed.

Table 1. Chemokine and chemokine receptor expression of tumors with and without TILs

| SYMBOL | No TILs (n = 30) | TILs (n = 27) | P** |
|--------|----------------|---------------|-----|
|        | n* (%) | Median | n* (%) | Median |     |
| CCL1   | 4 (13) | 0      | 5 (19) | 0      | 0.8 |
| CCL2   | 28 (93) | 16.9 | 26 (96) | 24.4 | 0.2 |
| CCL3   | 26 (87) | 7.5 | 27 (100) | 8.4 | 0.5 |
| CCL4   | 28 (93) | 10.9 | 27 (100) | 18.4 | **0.01** |
| CCL5   | 30 (100) | 59.5 | 27 (100) | 201.5 | **0.009** |
| CCL7   | 6 (20) | 0 | 4 (15) | 0 | 0.2 |
| CCL8   | 30 (100) | 24.4 | 27 (100) | 25.2 | 0.9 |
| CCL9   | 6 (20) | 0 | 5 (19) | 0 | 0.4 |
| CCL13  | 20 (67) | 3.5 | 17 (63) | 2.6 | 0.5 |
| CCL15  | 7 (23) | 0 | 3 (13) | 0 | 0.3 |
| CCL16  | 12 (40) | 0 | 16 (59) | 0.1 | 0.1 |
| CCL17  | 15 (50) | 0.1 | 13 (48) | 0 | 0.5 |
| CCL18  | 10 (33) | 0 | 10 (37) | 0 | 0.6 |
| CCL19  | 29 (97) | 7.7 | 27 (100) | 8.0 | 0.5 |
| CCL20  | 17 (57) | 2.1 | 21 (78) | 6.8 | **0.02** |
| CCL21  | 24 (80) | 3.9 | 23 (85) | 6.0 | 0.6 |
| CCL22  | 1 (3) | 0 | 2 (7) | 0 | 0.8 |
| CCL23  | 30 (100) | 31.7 | 27 (100) | 38.6 | 0.2 |
| CCL24  | 4 (13) | 0 | 3 (11) | 0 | 0.6 |
| CCL25  | 20 (67) | 1.2 | 16 (59) | 0.8 | 0.6 |
| CCL26  | 27 (90) | 6.3 | 27 (100) | 6.0 | 0.5 |
| CCL27  | 13 (43) | 0 | 15 (56) | 1.5 | 0.7 |
| CCL28  | 23 (77) | 16.9 | 24 (89) | 18.5 | 0.5 |
| CCR1   | 28 (93) | 7.3 | 26 (96) | 12.5 | 0.2 |
| CCR2   | 14 (47) | 0 | 12 (44) | 0 | 0.2 |
| CCR3   | 7 (23) | 0 | 4 (15) | 0 | 0.3 |
| CCR4   | 9 (30) | 0 | 7 (26) | 0 | 0.3 |
| CCR5   | 4 (13) | 0 | 12 (44) | 0 | 0.06 |
| CCR6   | 20 (67) | 3.1 | 16 (59) | 0.9 | 0.1 |
| CCR7   | 23 (77) | 6.2 | 23 (85) | 5.1 | 0.4 |
| CCR8   | 15 (50) | 0 | 7 (26) | 0 | 0.4 |
| CCR9   | 18 (60) | 1.1 | 21 (78) | 2.5 | 0.6 |
| CCR10  | 27 (90) | 7.7 | 21 (78) | 7.1 | 0.2 |
| CXCL1  | 10 (33) | 0 | 8 (30) | 0 | 0.3 |
| CXCL2  | 12 (40) | 0 | 14 (52) | 0.3 | 0.5 |
| CXCL3  | 18 (60) | 2.8 | 15 (56) | 0.2 | 0.4 |
| CXCL4  | 3 (10) | 0 | 2 (7) | 0 | 0.4 |
| CXCL5  | 3 (10) | 0 | 2 (7) | 0 | 0.9 |
| CXCL6  | 14 (47) | 0 | 13 (48) | 0 | 0.1 |
| CXCL8  | 29 (97) | 12.6 | 25 (93) | 10.5 | 0.6 |
| CXCL9  | 21 (70) | 4.6 | 18 (67) | 6.3 | 0.4 |
| CXCL10 | 30 (100) | 21.5 | 27 (100) | 18.6 | 0.9 |
| CXCL11 | 7 (23) | 0 | 23 (85) | 0 | 0.2 |
| CXCL12 | 28 (93) | 11.3 | 27 (100) | 20.0 | 0.5 |
| CXCL13 | 5 (17) | 0 | 7 (26) | 0 | 0.2 |
| CXCL14 | 30 (100) | 62.3 | 27 (100) | 61.4 | 0.2 |
| CXCL16 | 30 (100) | 173.6 | 27 (100) | 211.2 | 0.2 |
| CXCL17 | 26 (87) | 4.1 | 25 (93) | 4.9 | 0.4 |
| CXCR1  | 11 (7) | 0 | 12 (44) | 0 | 0.9 |
| CXCR2  | 30 (100) | 14.2 | 27 (100) | 9.9 | 0.5 |
| CXCR3  | 13 (43) | 0 | 17 (63) | 1.2 | 0.08 |
| CXCR4  | 16 (53) | 0.8 | 18 (67) | 3.7 | 0.2 |
| CXCR5  | 30 (100) | 11.6 | 27 (100) | 10.8 | 0.02 |
| CXCR6  | 20 (67) | 1.1 | 17 (63) | 1.6 | 0.3 |
| CXCR7  | 30 (100) | 63.0 | 26 (96) | 36.8 | 0.4 |
| CXCL1  | 26 (87) | 21.4 | 25 (93) | 19.5 | 0.7 |
| CXCL10 | 29 (97) | 13.6 | 27 (100) | 15.0 | 0.7 |
| CXCL11 | 14 (47) | 0 | 9 (33) | 0 | 0.2 |
| CXCL12 | 28 (93) | 3.2 | 22 (81) | 3.8 | 0.5 |

*Number of tumors with detectable expression.
**Adjusted P value, expression levels of tumors with vs. without TILs.
found to be associated with a favorable clinical outcome in patients with cutaneous melanoma administered a melanoma vaccine. Thus, characterization of TILs could lead to an improvement in the clinical efficacy of cancer immunotherapy, by permitting the selection of patients most likely to have a beneficial response. Further investigations of TILs may also help identify the main factors that make immune modulation ineffective and suggest specific manipulations. For example, CD8\textsuperscript{T} Tregs are potentially modifiable by blockade of LAG-3 interactions and a blocking LAG-3 immunoglobulin fusion protein is currently being evaluated in clinical trials.

Chemokine inhibitors, such as a CCL5 antagonist, may be potent immune-modifying agents with potential therapeutic benefit and are also under development.

Materials and Methods

**Uveal melanoma patient samples and pathological examination**

Tumors from 57 patients with uveal melanoma treated by enucleation at the Cleveland Clinic Cole Eye Institute were evaluated in this investigation. The study was approved by the Institutional Review Board, according to the tenets of the Declaration of Helsinki. Immediately following enucleation, transillumination was used to mark the tumor margins. Dissection was carried out through a scleral flap overlying the tumor base. Portions of each sample were processed for cryopreservation and genomic analyses, and the remainder of the eye was processed for conventional histopathology. The globe was fixed in 10% neutral buffered formalin and embedded in paraffin, after which hematoxylin and eosin-stained 4-μm sections were prepared. The tumor was examined for known histopathologic prognostic features for uveal melanoma, including the presence or absence of TILs, which was defined on the basis of more than 100 lymphocytes in 20 high power (40) fields.

**Gene expression array**

Pieces of fresh or frozen tumor tissue, 2 to 3 mm\textsuperscript{2}, were cut from the original sample and transferred immediately to 1 mL Trizol reagent. Total RNA was purified using a RNeasy MiniElute Cleanup Kit (Qiagen) as per manufacturer’s instructions. The concentration of the purified total RNA samples was measured using a Quant-It RiboGreen RNA Assay Kit (Molecular Probes, Inc.) and a fluorimeter. To synthesize first and second strand cDNA and amplify biotinylated cRNA from the total RNA, an Illumina Totalprep RNA Amplification Kit was used according to the manufacturer’s instructions. The purified cRNA samples were quantified to determine the volume required for the BeadChip hybridization step via the Quant-iT RiboGreen RNA Assay Kit. Hybridization to the Illumina Sentrix 8 BeadChip, which assesses 24,000 transcripts, was performed according to the manufacturer’s instructions without modification (Illumina). The Sentrix 8 BeadChips were read using an Illumina Beadarray reader.

**Immunohistochemistry**

IHC was performed on 4 μm sections of formalin-fixed paraffin-embedded tissues of selected cases using a Discovery XT automated stainer (Ventana Medical Systems). Antigen retrieval consisted of CC1 (Tris/Borate/EDTA buffer, pH 8.0–8.5) (Ventana Medical Systems) for 8 min at 95°C, 28 min at 100°C, and then an 8 min cool down to 37°C. The slides were then incubated with an anti-CD8 mouse monoclonal antibody (1:10 dilution, clone ID: 1A5, Catalog Number MU422-UC, Biogenex) for one hour at 37°C. A secondary antibody (UltraMap anti-Mouse AP) was applied for 12 min at 37°C. The chromogenic substrate (ChromoMap Fast Red) was applied for 16 min at 37°C after 4 min of Activator Red and Naphthol. Slides were
counterstained with Hematoxylin II. A similar process for FOXP3 was performed using anti-FOXP3 mouse monoclonal antibody (1:50 dilution, clone ID: 236A/E7, Catalog Number 14–4777-82, eBIOSCIENCES) except that the anti-FOXP3 was applied for one hour. RANTES/CCL5 antibody (1:100 dilution, rabbit polyclonal antibody, P20, catalog number 2988, Cell Signaling) was applied for 12 hours at 25°C. A secondary antibody (UltraMap anti-rabbit AP) was applied for 32 min at 37°C. Next, 4 min of combined Activator Red and Naphthol were applied and then rinsed off. The chromogenic substrate (ChromoMap Fast Red) was applied for 16 min at 37°C. Slides were counterstained with Hematoxylin II.

Data analysis
Analysis and normalization of expression data were carried out using BeadStudio 2.0 (Illumina). Expression intensities of 91 lymphocyte-associated factors were evaluated. Expression intensity below background in a sample was considered to be absent (0). Error bars represent standard error. Differences in expression intensities between tumors with and without TILs were analyzed using 2-sided Student’s t tests after log transformation. The P-values were adjusted for multiple testing according to the methods of Benjamini and Hochberg. Comparisons that exhibited an adjusted P-value < 0.05 were called differentially expressed. Correlations were assessed using Spearman rank correlations and P < 0.05 was considered statistically significant.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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