The Signature of Serum Modified Nucleosides in Uveitis

Haoze Zhou,[1] Yiqiu Hu,[2] Guangming Qin,[1] Jinfeng Kong,[1] Xiujuan Hong,[2] Cheng Guo,[2] Jian Zou,[1,3] and Lei Feng[1]

[1]Eye Center, Second Affiliated Hospital, School of Medicine, Zhejiang University, Hangzhou, Zhejiang, China
[2]Cancer Institute (Key Laboratory of Cancer Prevention and Intervention, China National Ministry of Education), The Second Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou, Zhejiang, China
[3]Institute of Genetics, Zhejiang University International School of Medicine, Hangzhou, China

Correspondence: Cheng Guo, Cancer Institute (Key Laboratory of Cancer Prevention and Intervention, China National Ministry of Education), The Second Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou, Zhejiang 310009, China; cheng_guo@zju.edu.cn.
Jian Zou, Institute of Genetics, Zhejiang University International School of Medicine, Hangzhou 310029, China; jianzou@zju.edu.cn.
Lei Feng, Eye Center, Second Affiliated Hospital, School of Medicine, Zhejiang University, Hangzhou, Zhejiang 310009, China; leifeng@zju.edu.cn.

HZ, YH, and GQ contributed equally to this work.

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Purpose. This study aims to evaluate the metabolism of serum-modified nucleosides in uveitis by using liquid chromatography-tandem mass spectrometry (LC-MS) and to develop potential diagnostic biomarkers for uveitis.

Methods. Forty-two patients with different subtypes of uveitis (idiopathic uveitis, Vogt-Koyanagi-Harada [VKH] disease, and ankylosing spondylitis [AS]) and 32 healthy controls were recruited in this retrospective case-control study. The concentrations of 23 modified nucleosides in patient serum were quantified by LC-MS. The data was statistically analyzed with SPSS and GraphPad Prism.

Results. The data revealed that 13 out of 23 modified nucleosides (m^6A, m^1A, m^6A_m, C_m, ac^4C, G_m, m^1G, m^2G, $m^{2,2}G$, U_m, m^3U, m^5U, and m^5U_m) effectively showed quantifiable chromatographic peaks. The statistical results indicated that there were extremely significant differences for m^2G, G_m, C_m, and m^1G between healthy controls and uveitis patients. The differences for G_m, m^6A,and m^5U were able to further assort idiopathic uveitis and uveitis with systemic inflammation including VKH and AS. Interestingly, each specific subtype of uveitis is characterized by its signature combination of serum-modified nucleotides comparing with healthy controls.

Conclusions. This study revealed that the metabolism of serum-modified nucleosides in uveitis patients display significant differences from healthy controls. The signature combination of serum modified nucleotides for each subtype of uveitis may be applied for the potential diagnosis of uveitis.

Keywords: uveitis, serum modified nucleosides, LC-MS/MS, VKH, ankylosing spondylitis

Uveitis is a heterogeneous disease characterized by inflammation of the uveal tract, which includes the iris, ciliary body, and choroid, often affecting the retina and its blood vessels.[1,2] Uveitis is one of the leading causes of visual impairment, accounting for approximately 10% to 15% of blindness cases in developed countries.[3–6] Improper diagnosis and treatment of uveitis may lead to blindness.[2] The pathophysiology of uveitis is complicated, including infectious and noninfectious causes.[3] Noninfectious uveitis represents the majority of cases.[3,7] Many causes may lead to noninfectious uveitis, including uveitis associated with systemic immune abnormalities and idiopathic uveitis. The different types of uveitis may convey differences in pathogenic mechanisms and inflammatory responses and the need for pertinent medical treatments. Marked by the specific clinical characteristics and the connections with the systemic symptoms, uveitis with systemic inflammation could be categorized into many types,[1,8] such as Vogt-Koyanagi-Harada (VKH) disease and ankylosing spondylitis (AS). VKH is characterized by acute, diffuse, and bilateral uveitis associated with neurological (meningeal), auditory, and integumentary manifestations.[9] AS is a chronic inflammatory disease that mainly affects the axial skeleton, with uveitis as the most frequent extra-articular manifestation.[10]

Immune responses, both innate immune and adaptive immune, are known to play a pivotal role in the pathogenesis of uveitis.[11,12] Many studies have reported that immune and inflammatory factors, such as interleukin (IL)-1β, IL-6, interferon (IFN)-γ, and tumor necrosis factor (TNF)-α, are highly active in uveitis.[11,13–15] Furthermore, the noninfectious uveitis is often associated with autoimmune and autoinflammatory processes.[9,16–18] However, the complication of immune responses in uveitis, characterized by diverse cytokines and immune cells activation,[16] challenges the precision diagnosis and therapies of uveitis.

In the past few decades, growing evidence has identified the modifications of RNA molecules as a form of epigenetic regulation, which is a critical player in multiple biological processes of various diseases, especially in the regulation of immune responses.[19–23] Up to now, more

than 170 types of RNA modifications have been identified.[21,24–27] The RNA modifications influence various aspects of RNA metabolism in immune cells, including stability, splicing, and translation, which in turn affects immune responses.[28–30] For instance, m^6A and m^1A modifications have been linked to the regulation of macrophage polarization, a key process in innate immunity.[31,32] Modifications to m^6A_m and U_m were associated with modulating protein synthesis and RNA stability, thereby influencing immune cell function and the host's defense mechanisms.[33,34] Moreover, the oncogenic role of NAT10/ac^4C/FOXP1 axis in initiating crosstalk between cancer cell glycolysis and immunosuppression has been revealed.[35]

Despite the recognized importance of RNA modifications in immune regulation, the RNA modifications in uveitis remain unexplored. In this study, 23 types of nucleosides modification-derived from all types of nucleosides (A, G, C, U) were quantified in serum samples of noninfectious uveitis patients by LC-MS/MS. The results revealed that the abundance of serum modified nucleosides in uveitis patients display significant differences from healthy controls. Significantly, each specific subtype of uveitis can be sorted out by its signature combination of serum modified nucleotides. These signature combinations could be a potential biomarker of precision diagnosis of uveitis.

Material and Methods

Subject Recruitment and Sample Collection

This study adhered to the tenets of Declaration of Helsinki was approved by the Ethics Committee of the Second Affiliated Hospital, Zhejiang University School of Medicine. All participants underwent ophthalmic examinations including digital slit lamp microscopy, ocular ultrasound scanning, fundus fluorescein angiography, and optical coherence tomography. Patients with active uveitis who had not received systemic treatment such as systemic steroid therapy and immunosuppressive agent in six months were included. Exclusions to the enrollment were as follows: diabetes mellitus, hypertension, dyslipidemia, history of other autoimmune disease, immunodeficiency, and other disorders.

A total of 32 healthy controls (mean age 48.28 ± 17.46 years, range 23–84 years) and 42 patients with uveitis (mean age 42.40 ± 13.76 years, range 20–69 years) were recruited. The diagnosis of uveitis was made by two senior ophthalmologists independently. All the participants signed the informed consent forms.

In this study, the anatomical classification of uveitis was done using the standardized nomenclature of uveitis.[36] The diagnosis of Vogt-Koyanagi-Harada (VKH) disease followed the international committee revised VKH disease diagnostic criteria.[37] Ankylosing spondylitis (AS) was diagnosed according to the modified New York Criteria.[38] The diagnostic criteria for idiopathic panuveitis includes (1) inflammation involving iris, ciliary body, and choroid; (2) exclusion of various infectious factors; and (3) the potential etiology cannot be classified by the standardized nomenclature of uveitis[36] through the collection of detailed medical history and series of examinations such as HLA-B27, T-spot test and chest radiography. Serum samples were collected from all patients and controls and then stored at −80°C before further processing.

Chemicals and Reagents

The analytical standards of m^6A, m^1A, m^6A_m, C_m, ac^4C, m^1G, m^2G, G_m, $m^{2,2}G$, m^3U, m^5U, U_m, and m^5U_m and their isotope-labeled internal standards (IS) including D_3-N^6-methyladenosine ($[D_3]m^6A$), D_3-N^1-methyladenosine ($[D_3]m^1A$), D_3-N^6-2′-O-dimethyladenosine ($[D_3]m^6A_m$), $[D_3]$-2′-O-methylcytidine ($[D_3]C_m$), $^{13}C_5$-N^4-acetylcytidine ($[^{13}C_5]ac^4C$), D_6-N^2,N^2-dimethylguanosine ($[D_6]m^{2,2}G$), $^{13}C_5$-5-methyluridine ($[^{13}C_5]m^5U$), and D_3-2′-O-methyluridine ($[D_3]U_m$) were purchased from Toronto Research Chemical (Toronto, ON, Canada). Chromatographic grade acetonitrile (CH_3CN) was purchased from Merck KGaA (Darmstadt, Germany). Formic acid (HCOOH) was obtained from Fluka (Muskegon, MI, USA). Ammonium formate ($HCOONH_4$) and malic acid were ordered from Sigma-Aldrich (St. Louis, MO, USA). Water used throughout the study was purified by a Milli-Q purification apparatus (Millipore, Milford, MA, USA).

Sample Pretreatment

Serum samples 40 µL were naturally thawed in ice and spiked with 5 µL of isotope-labeled internal standards (IS). Then 135 µL of prechilled methanol/acetonitrile (1:2, v/v) was added to remove the protein. The obtained mixture was placed at −20°C for two hours after one minute of vortex. Subsequently, the mixture was spun in a centrifuge at 13,000 rpm at 4°C for 15 minutes. Next, 144 µL of supernatant was evaporated to dryness under vacuum. Then, 32 µL of acetonitrile/water (9:1, v/v) was used to redissolve the dried samples. After vortex for 10 seconds, ultrasonication for 15 seconds, and centrifuging at 13,000 rpm for 15 minutes at 4°C, 25 µL of the supernatant fraction was aspirated into the vial for LC-MS/MS detection.

LC-MS/MS Analysis

Chromatographic separation was carried out by using a Waters BEH Amide column (2.1 mm × 100 mm, 1.7 µm). Analysis of the samples was performed on a LC-MS/MS system consisting of an Acquity UPLC system (Waters, Milford, MA, USA), and a 4000 QTRAP mass spectrometer (AB SCIEX, Foster City, CA, USA) equipped with an electrospray ionization source in positive-ion mode. Data acquisition and processing were controlled by Analyst 1.6.3 software. The mobile phases were (A) H_2O containing 0.2% formic acid, 10 mM ammonium formate and 0.05 mM malic acid, and (B) acetonitrile containing 0.2% formic acid, 2 mM ammonium formate, and 0.05 mM malic acid. The desired sample separation was achieved by the optimized LC gradient program as follows: 0-5.5 minutes, 95% B; 5.5–7 minutes, 95%–92% B; 7–9 minutes, 92%–85% B; 9–9.5 minutes, 85%–83% B; 9.5–11 minutes, 83%-80% B; 11–11.5 minutes, 80%-95% B; 11.5–15 minutes, 95% B. The flow rate was 0.3 mL/min. The samples were stored at 4°C and the column temperature was set at 30°C. The injection volume was 5 µL.

Multiple reaction monitoring (MRM) mode was used to quantify these modified nucleosides by monitoring the corresponding ion transitions. The ion source temperature, spray voltage, gases 1 and 2 and curtain gas were set at 550°C, 5.5 kV, 45 psi, 45 psi and 50 psi, respectively.

The calibration curves were made by comparing the peak area ratio and concentration ratio of the analyte/IS. The calibration curves were established as $y = ax + b$ by plotting the peak area ratios of the nucleosides to the corre-

sponding IS (y) versus the concentration of the analyte (x). The final concentrations of IS were as follows: 25 nM of $[D_3]m^6A$, 1250 nM of $[D_3]m^1A$, 125 nM of $[D_3]m^6A_m$, 500 nM of $[^{13}C_5]C_m$, 500 nM of $[^{13}C_5]ac^4C$, 500 nM of $[D_6]m^{2,2}G$, 500 nM of $[D_3]U_m$, and 500 nM of $[^{13}C_5]m^5U$. Since the isotope-labeled m^1G, m^2G, G_m, m^3U and m^5U_m were not commercially available, we used the IS with similar structures to build their calibration curves. For m^1G, m^2G and G_m, $[D_6]m^{2,2}G$ was used as the IS. And for m^3U and m^5U_m, $[D_3]U_m$ was used as IS.

Statistical Analysis

Statistical analysis of data was performed using IBM SPSS Statistics 24.0 software (IBM, Armonk, NY, USA) and GraphPad Prism (GraphPad, La Jolla, CA, USA). The differences of analytes concentration between healthy controls and uveitis patients were accessed by Student's *t*-test or ordinary one-way analysis of variance (ns ($P > 0.05$) represented not significant; * ($0.01 < P < 0.05$) and ** ($0.001 < P < 0.01$) represented significant; *** ($0.0001 < P < 0.001$) and **** ($P < 0.0001$) represented extremely significant). Besides, we carried out receiver operating characteristic (ROC) analysis to assess the ability of these nucleosides to discriminate uveitis patients from healthy controls.

Results

Diagnosis of Patients

In this study, a total of 32 healthy controls and 42 patients with uveitis were enrolled. The characteristics of the study population are listed in Table 1. 10 patients were conformed to the international committee revised VKH diagnostic criteria.[37] 12 patients were diagnosed as AS associated uveitis according to the modified New York Criteria.[38] The other 20 patients were attributed to idiopathic panuveitis according to the following diagnosis criteria: (1) inflammation involving iris, ciliary body and choroid; (2) exclusion of various infectious factors; (3) the potential etiology cannot be classified by the standardized nomenclature of uveitis[36] through the collection of detailed medical history and series of examinations such as HLA-B27, T-spot test and chest X-Ray. All patients were newly diagnosed and received no therapies in 6 months before enrolled in the study. Participants were excluded when gathering systemic diseases such as cancer, diabetes and hypertension.

In general, the patients enrolled in this study had typical uveitis symptoms, such as eye pain, tearing, redness, light sensitivity, floaters and blurred vision. The results of examinations included hypopyon and flocculent exudation in the pupil region shown by slit lamp-microscope examination, macular edema shown by OCT, severe vitreous opacity shown by ocular ultrasound, optic disc hyper fluorescence, macular leakage and capillary leakage at the posterior pole retina shown by FFA images and so on supporting the specific diagnosis (Fig. 1). Additionally, there was no statistically significant difference in age and sex distribution between the disease group and the normal group by student's t-test (Table 1).

Detection of Modified Nucleotides by LC-MS/MS

These modifications, increasingly recognized as pivotal in gene expression regulation, have drawn analogies to the

Table 1. Basic Characteristics of Patients and Controls

Variables	Healthy Controls	Vogt-Koyanagi-Harada Disease	Ankylosing Spondylitis Associated Uveitis	Idiopathic Panuveitis	Total Uveitis	*P* Value
Number of groups	32	10	12	20	42	
Age (years)	42.45 ± 15.64	47.70 ± 10.43	37.17 ± 11.65	48.28 ± 17.46	42.89 ± 15.45	0.105
Sex						0.339
Male	15 (46.9%)	3 (36.4%)	6 (50.0%)	6 (26.7%)	15 (36.8%)	
Female	17 (53.1%)	7 (63.6%)	6 (50.0%)	14 (73.3%)	27 (63.2%)	
History of drug treatment	No	No	No	No	No	

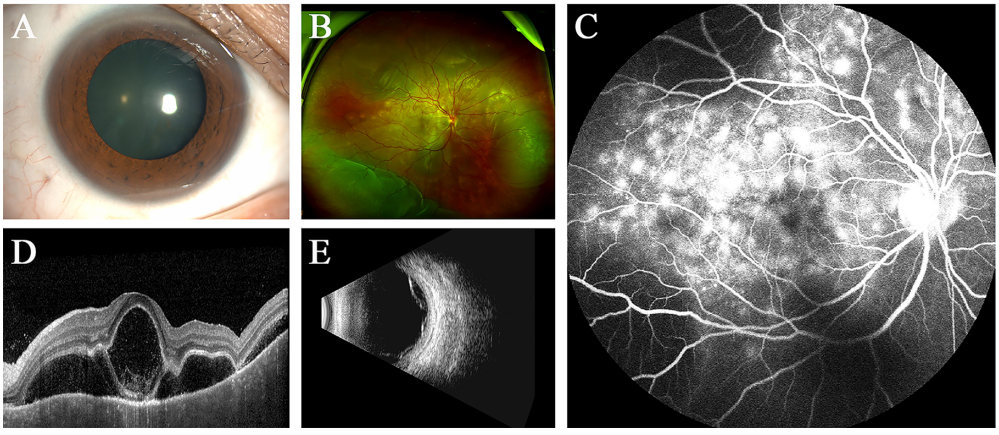

Figure. 1. The images of one uveitis patients with early-stage Vogt-Koyanagi-Harada disease in this study. **(A)** Slit-lamp bio microscopy of the anterior segment showed no abnormalities. **(B)** Ultra-wide field fundus photograph of papilledema and multiple focal protrusions in the posterior pole. **(C)** Fluorescein angiography of multiple leakage spots and large vascular leakage areas with optic neuritis, demonstrating multiloculated appearance. **(D)** Optical coherence tomogram of an exudative retinal detachment, demonstrating septate appearance. **(E)** Ocular ultrasound image of vitreous opacity.

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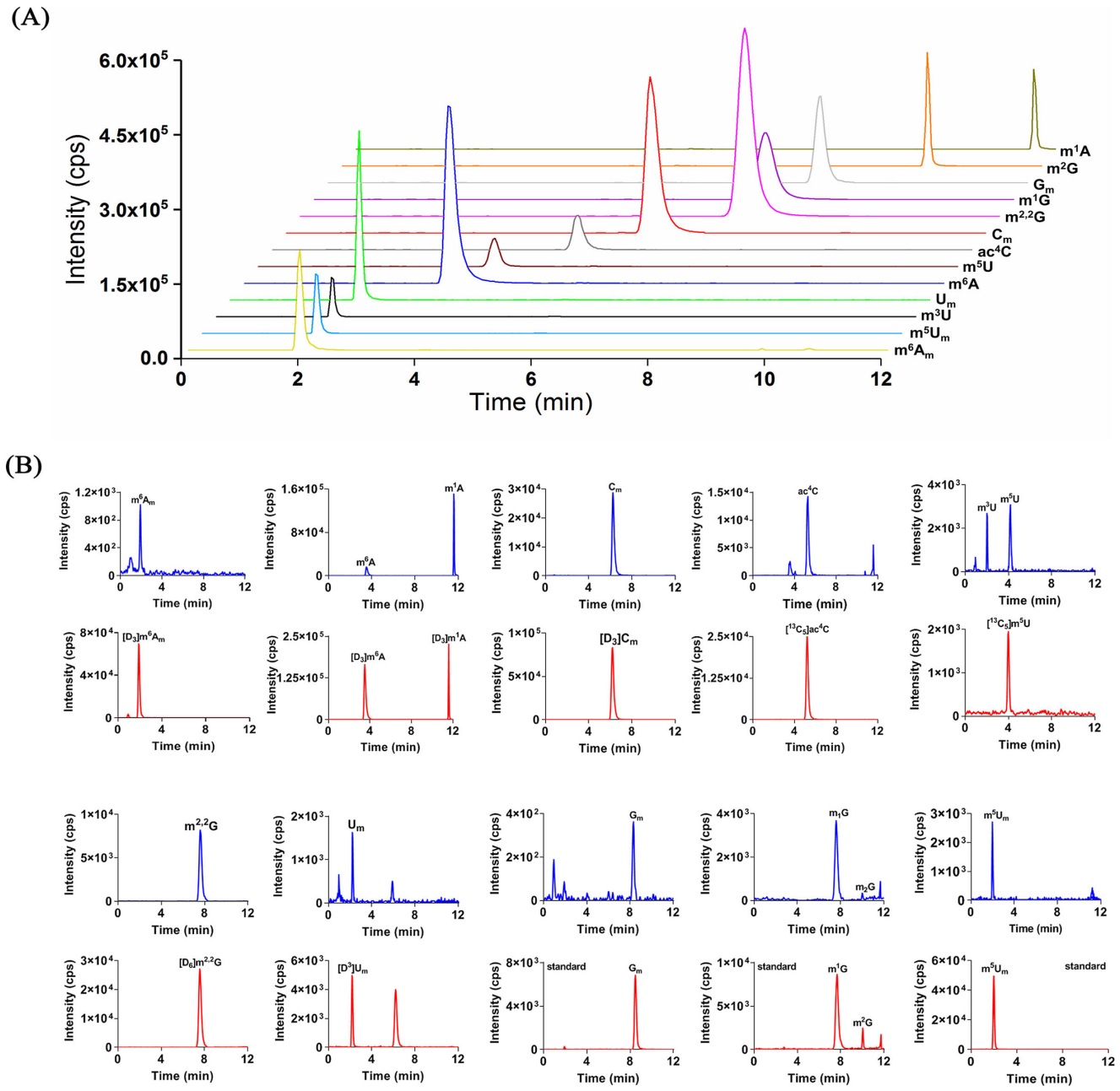

Figure 2. **(A)** The MRM chromatograms of modified nucleosides standards. **(B)** Identification of modified nucleosides in human serum. The MRM chromatograms of these 13 modified nucleosides in a serum sample of a healthy control are shown as the *blue curves* with each *red curve* below, which represents the MRM chromatograms of spiked stable isotope-labeled internal standards or standards. (The last three red curves represented standards, because there are no specific isotope-labeled internal standards for G_m, m^1G, m^2G and m^5U_m commercially available.)

epigenetic landscape sculpted by DNA and histone alterations, leading to the coining of terms such as 'RNA epigenetics'. LC-MS/MS has been proved to be a powerful analytical platform for evaluating the modified nucleosides in serum samples due to its great advantages in selectivity, sensitivity and accuracy.[39–45]

In the LC-MS/MS measurement, clear chromatographic separation and proper chromatographic peak shape were obtained after the chromatographic conditions and the MRM parameters were optimized. Besides, the calibration curve of each analyte showed excellent linearity (R2 > 0.999) (Supplementary Table S1). The concentration of the nucleoside modifications in serum samples was calculated according to the calibration curves.

Twenty three types of modified nucleosides with commercially available standards were detected by LC-MS/MS (Supplementary Fig. S1). 10 of them (A_m, $m^6{}_2A$, hm^6A, m^6G, m^7G, m^3C, m^5C, m^5C_m, hm^5C, ψ) did not show quantifiable chromatographic peaks, indicating their low abundance in serum. The other 13 types of modified nucleosides (m^6A, m^1A, m^6A_m, C_m, ac^4C, G_m, m^1G, m^2G, $m^{2,2}G$, U_m, m^3U, m^5U and m^5U_m) were successfully detected and

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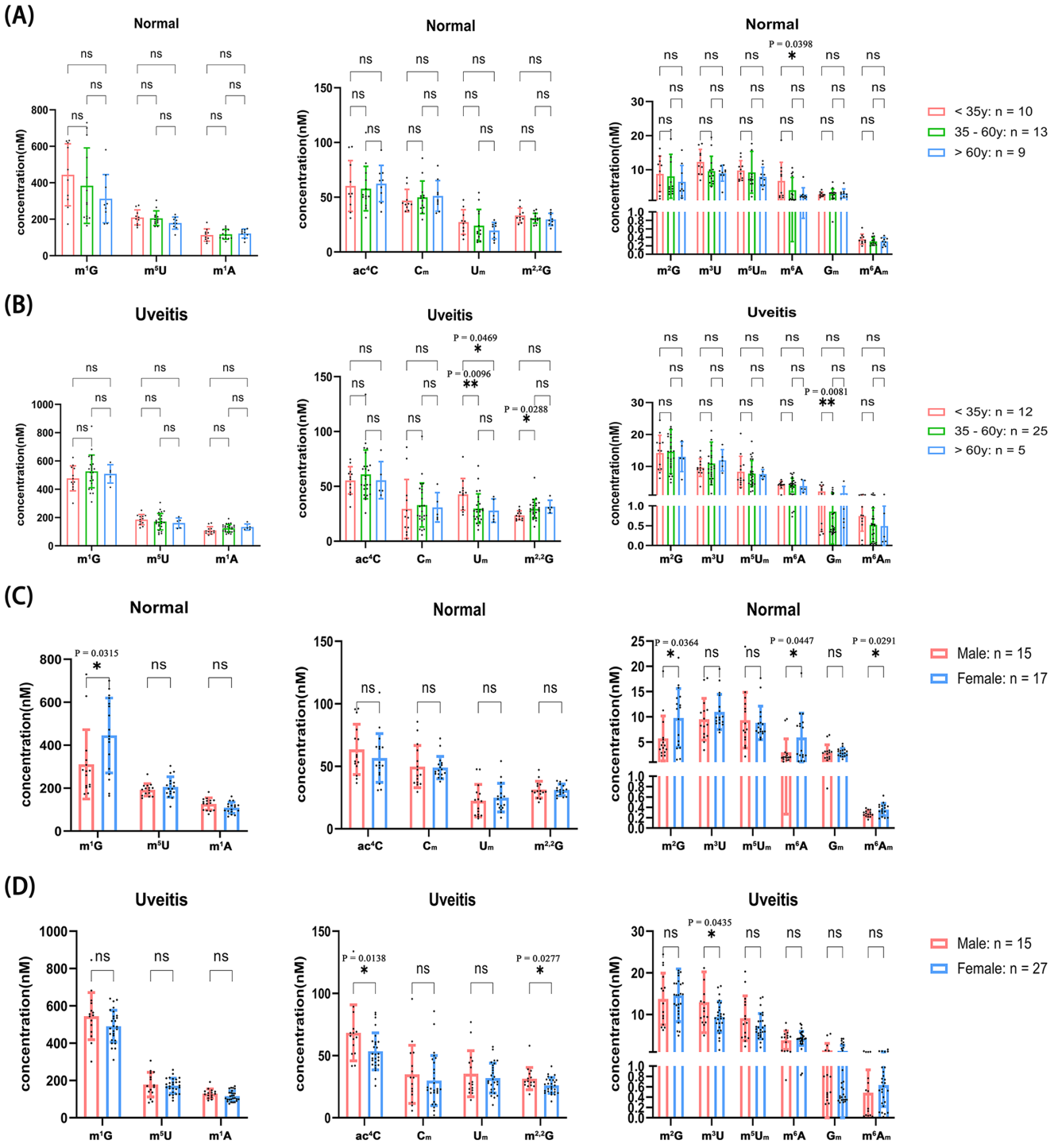

Figure 3. **(A)** Comparation of concentration of modified nucleosides in normal people between ages; **(B)** Comparison of concentration of modified nucleosides in patients with uveitis between ages; **(C)** Comparison of concentration of modified nucleosides in normal people between sexes; **(D)** Comparison of concentration of modified nucleosides in patients with uveitis between sexes.

quantified in serum examples of patients with uveitis and healthy controls (Fig. 2). Of note, all of these modified nucleosides detected in serum showed the same retention time as the corresponding IS or standards, further confirming the presence of these modified nucleosides in human serum (Fig. 2B).

The Comparison of the Concentration of Serum-Modified Nucleosides Between Ages and Sexes

A standard curve was drawn for each modified nucleosides and sample concentration was determined based on the specific standard curve. We observed that the concentration

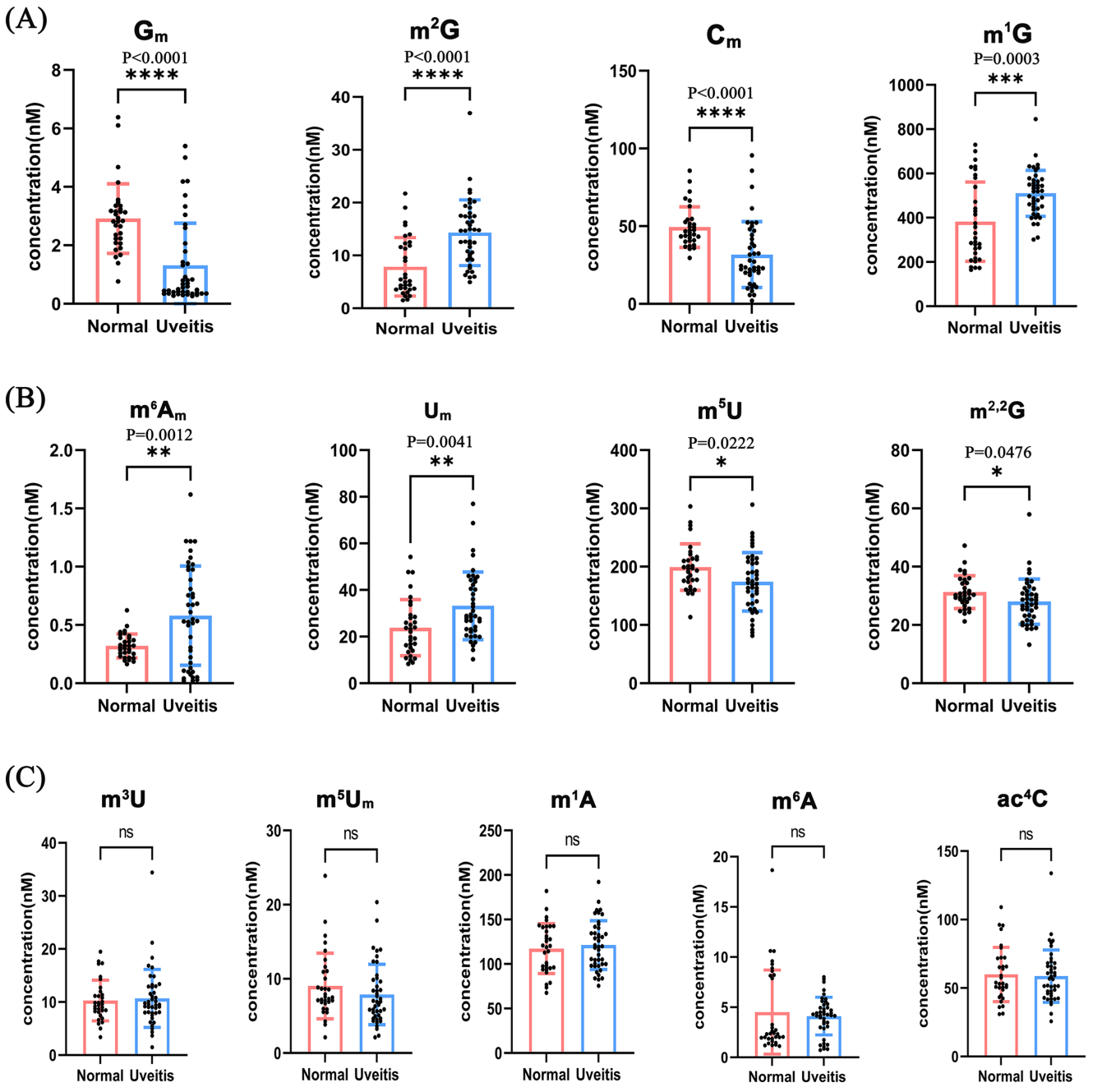

Figure 4. The measured concentrations of modified nucleosides in serum samples from normal controls and uveitis patients and statistical analysis. **(A)** Modified nucleosides that showed the most significant difference. **(B)** Modified nucleosides that showed significant difference. **(C)** Modified nucleosides that showed no significant difference. (The values represent the mean ± standard deviation. Significance was determined using unpaired Student's t-test. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ and ****$P < 0.0001$.)

of individual modified nucleosides varied in human serum (Fig. 3). The concentrations of m^1A, m^1G and m^5U were higher than 100 nM, the concentrations of C_m, ac^4C, $m^{2,2}G$ and U_m were between 20 nM and 100 nM, and the concentrations of m^6A, m^6A_m, G_m, m^2G, m^3U and m^5U_m were between 0.2 nM and 20 nM.

Before analyzing the characteristic of uveitis, we first tested whether there was significant difference between ages and sexes. All patients and healthy controls were divided into 3 groups by age (<35 years, 35–60 years, >60 years) for the statistical analyses. The results showed that only a few types of serum modified nucleosides displayed significant difference between ages (Figs. 3A–B). For instance, the concentration of m^6A in the <35 y healthy group was significantly higher than in the >60 y healthy group (Fig. 3A). In uveitis patients, the concentration of $m^{2,2}G$, U_m and G_m showed significantly differences between the <35 y group and the 35-60 y group. Moreover, the concentration of U_m was statistically higher in the <35 y patient group than in the >60 y patient group (Fig. 3B). No statistically significant differences were observed for the other age-based comparison analyses (Figs. 3A and 3B).

Similarly with the age-based analyses, we tested whether there was significant difference between sexes. Consistently, the statistical results showed that only a few types of serum modified nucleosides displayed significant difference between sexes (Figs. 3C–D). Specifically, the concentration of m^1G, m^6A, m^6A_m and m^2G in healthy male group was significantly lower than in the female healthy group, and the concentration of ac^4C, $m^{2,2}G$ and m^3U in the patient male group was significantly higher than in the patient female group (Fig. 3D).

We further examined the serum nucleoside modifications by categorizing the participants according to both age and gender. Consistently, several serum modified nucleosides displayed significant difference between ages and genders, although specific modifications showed difference when analyzed within different demographic categories (Supplementary Figs. S2–S3).

Taking together, the above results showed that a few types of serum modified nucleosides displayed significant difference between ages and sexes.

Differentiate Between the Patients With Uveitis From the Healthy Controls by the Concentration of Serum-Modified Nucleosides

We next evaluated whether there were differences in the concentrations of these modified nucleosides between the patients with uveitis and the healthy controls. Interestingly, we observed that most of the concentrations of serum modified nucleosides display significant differences between uveitis patients and healthy controls (Fig. 4). Specifically, the concentration of m^2G, G_m, C_m and m^1G showed extremely significant differences (p value < 0.001) (Fig. 4A), the concentration of m^6A_m, U_m, m^5U and $m^{2,2}G$ showed significant differences (0.001 < p value < 0.05) (Fig. 4B), and the concentration of m^3U, m^5U_m, m^1A, m^6A, and ac^4C showed no significant difference (p value > 0.05) (Fig. 4C) between patients and controls. Furthermore, compared with healthy controls, the concentrations of m^2G, m^1G, m^6A_m, U_m were significantly upregulated, while, the concentrations of G_m, C_m, m^5U and $m^{2,2}G$ were significantly downregulated (Figs. 4A and 4B). These results indicated that the abundance of serum modified nucleosides in patients with uveitis has significant changes. The signature combination of serum modified nucleotides may be applied for the potential biomarkers of uveitis diagnosis.

ROC Analysis for These Nucleosides in Serum From Uveitis Patients and Healthy Controls

We further constructed multivariate binary logistic regression to evaluate the diagnostic potential of G_m, m^2G, C_m, m^1G, m^5U, U_m, $m^{2,2}G$ and m^6A_m for uveitis. Moreover, we derived the ROC curves and obtained the area under the curve (AUC) (Fig. 5). The AUC for G_m, m^2G, C_m, m^1G, m^5U, U_m, $m^{2,2}G$ and m^6A_m, were 0.8274, 0.8028, 0.7879, 0.7180, 0.7180, 0.7009, 0.6629, and 0.6585, respectively. Moreover, when all these eight serum-modified nucleosides were considered, the diagnostic AUC value for uveitis could reach 0.9903 (Fig. 5), which showed better diagnostic potency than single modified nucleoside. These results support that these modified nucleosides can be regarded as potential indicators for the precision diagnosis of uveitis.

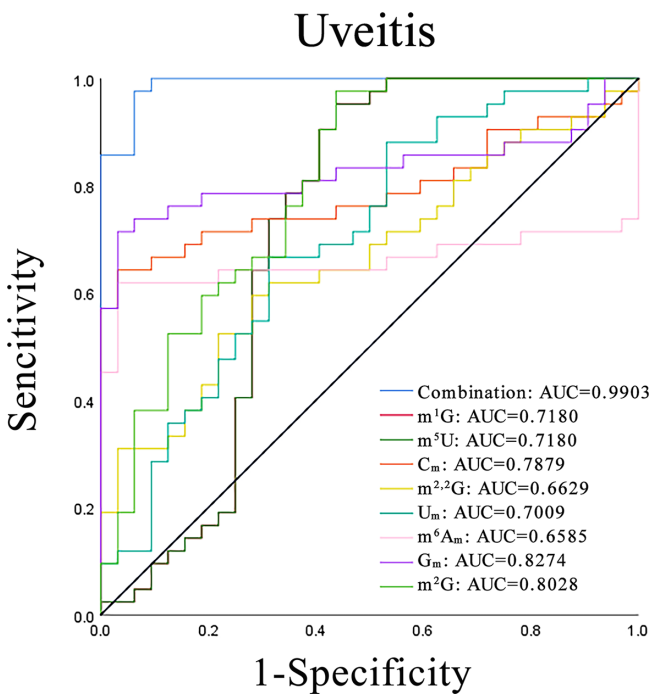

Figure 5. The receiver operating characteristic (ROC) analysis for m^1G, m^5U, C_m, $m^{2,2}G$, U_m, m^6A_m, G_m, m^2G and the combination of them in serum samples of uveitis patients.

The Difference of the Concentration of Serum Modified Nucleosides Between the Idiopathic Uveitis and Uveitis With Systemic Inflammation

Uveitis associated with systemic immune abnormalities and idiopathic uveitis may convey differences in pathogenic mechanisms and inflammatory responses. Indeed, the result manifested that, compared with systemic inflammation (VKH + AS), the decreased levels of G_m and the increased levels of m^6A and m^5U were exhibited in idiopathic uveitis (Fig. 6A). Additionally, the concentration of G_m ($P < 0.0001$) and m^6A ($P = 0.0009$) showed extremely significant differences, and the concentration of m^5U ($P = 0.0071$) showed significant differences between idiopathic uveitis and systemic immune diseases (Fig. 6A). Although the means of m^6A_m, m^1A, C_m, and m^5U_m seemed to be different, no statistical difference was presented in the other 10 modified nucleosides including them ($P > 0.05$) (Fig. 6B).

The Signature Combination of Serum-Modified Nucleosides Between the Subtypes of Uveitis

We next evaluate whether different subtypes of uveitis are characterized with specific combination of serum modified nucleotides. We statistically analyzed the profiles of 13 modified nucleosides in patients with three subtypes of uveitis (idiopathic uveitis, VKH disease, and AS) compared with healthy controls. Notably, signature combinations of serum modified nucleotides may define the subtypes of uveitis (Fig. 7, Table 2). Specifically, extremely significant differences in C_m and m^5U ($P < 0.001$) and significant differences in m^1G, $m^{2,2}G$, U_m, and G_m ($0.001 < P < 0.05$) were observed between idiopathic uveitis patients

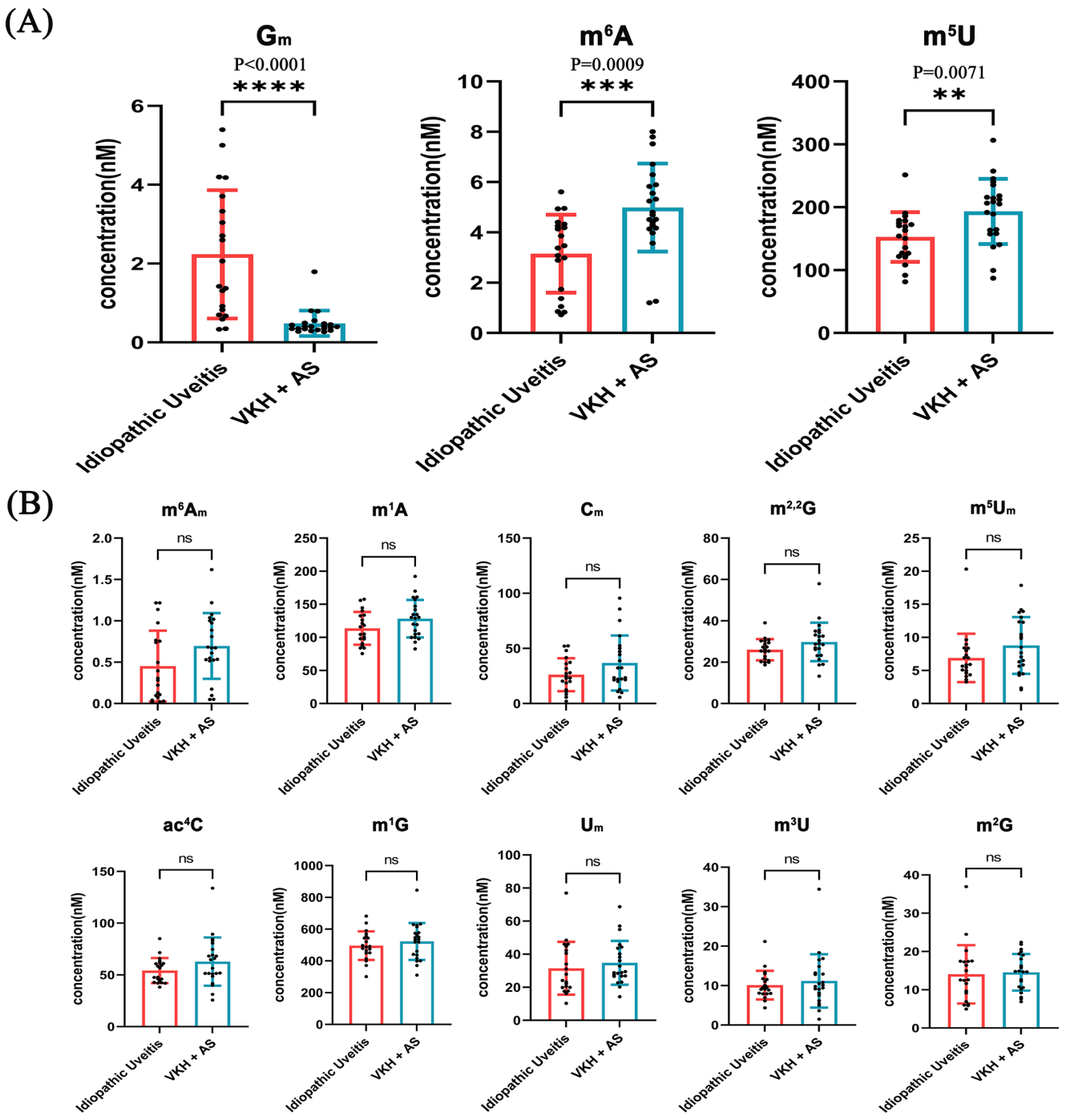

Figure 6. The comparison of serum concentrations of 13 types of modified nucleosides between patients with idiopathic uveitis and those with systemic inflammatory conditions (VKH disease and AS). **(A)** 3 modified nucleosides with significant difference between idiopathic uveitis group and systemic group. **(B)** 10 modified nucleosides with no significant difference between idiopathic uveitis group and systemic group. (The values represent the mean ± standard deviation. Significance was determined using unpaired Student's *t*-test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ and $^{****}P < 0.0001$.)

and healthy controls (Fig. 7A). In VKH disease patients, extremely significant deviations were detected in m^2G, G_m, C_m, and U_m ($P < 0.001$) and significant differences in m^1G and m^6A_m ($0.001 < P < 0.05$) compared with controls (Fig. 7B). In AS patients, extremely significant deviations were detected in G_m and m^6A_m ($P < 0.001$) and significant differences in m^1G and m^2G ($0.001 < P < 0.05$) compared with controls (Fig. 7C). Consistent with the results showed in Figure 4C, the modified nucleosides m^3U, m^5U_m, m^1A, m^6A, and ac^4C exhibited no significant deviations between uveitis patients and healthy individuals (Fig. 7D). These findings underscored the heterogeneity in modified nucleoside profiles among the subtypes of uveitis, and suggest that specific combinations of these biomarkers could serve as potential diagnostic indicators to differentiate subtypes of uveitis.

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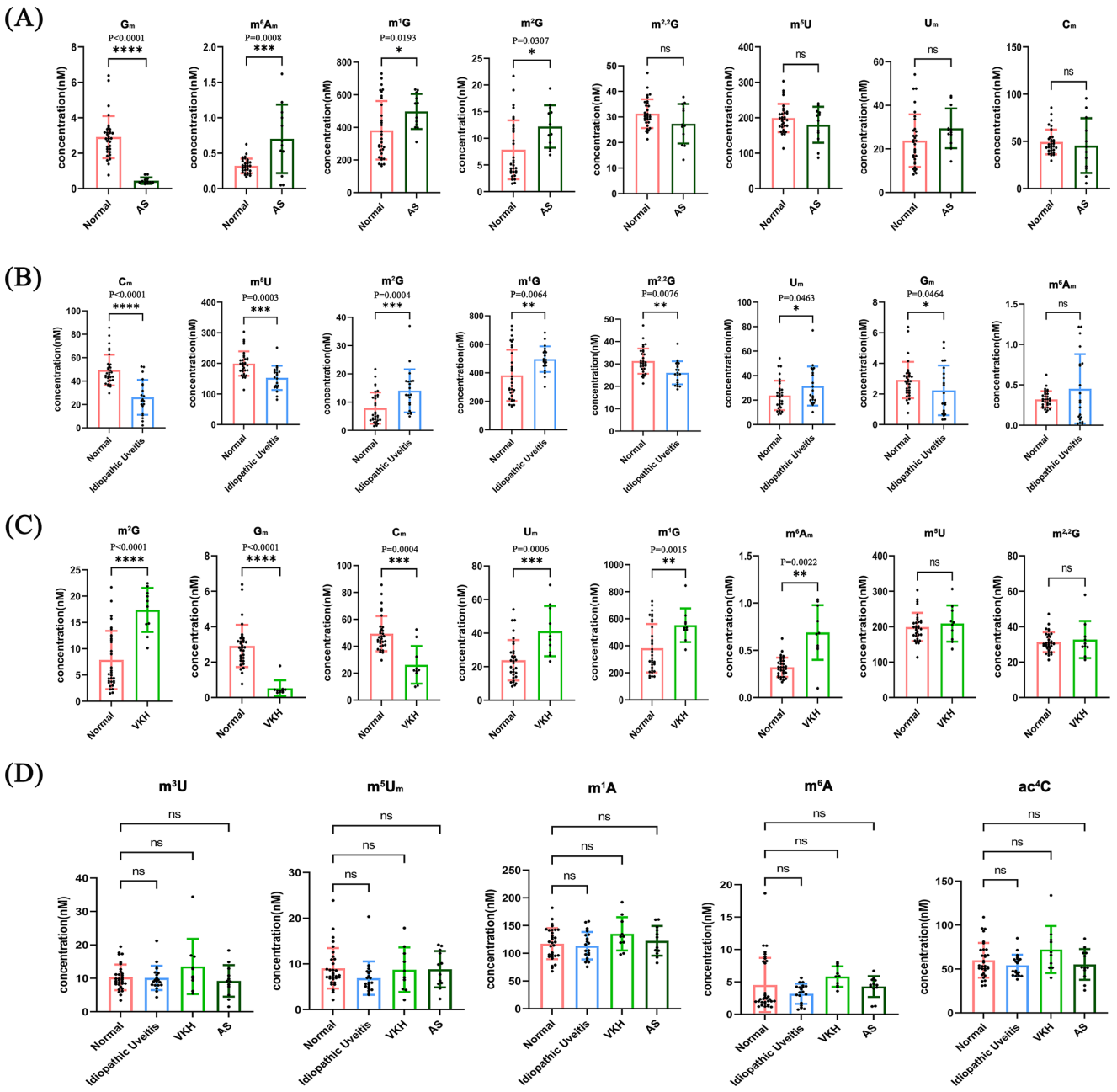

Figure 7. **(A–C)** The measured concentrations of modified nucleosides in serum samples from patients with each subtype of uveitis and normal controls. **(D)** The five modified nucleosides showing no significant difference between uveitis patients and the healthy controls. (The values represent the mean ± standard deviation. Significance was determined using ordinary one-way ANOVA. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ and ****$P < 0.0001$.)

Discussion

Uveitis represents an intraocular inflammatory disease with complex etiologies that pose a significant risk of blindness.[1,8] The Standardization of Uveitis Nomenclature Working Group, an international collaboration dedicating to improving research in the field of uveitis,[36] developed classification criteria for the 25 of the most common subtypes of uveitis. The characteristic signature of serum modified nucleosides in various subtypes of uveitis confirms the complicate pathogenesis of uveitis, and may serve as a potential biomarker for the early detection of uveitis. However, according to the current diagnosis criteria, similar clinical manifestation can result from different underlying immune reactions in some uveitis patients, which causes unresponsiveness to therapies.[46] The early precision diagnosis and therapies of uveitis are needed to benefit the patients by preventing the severer consequences. In this study, 23 types of modified nucleosides with commercially available standards were quantitatively analyzed in serum of uveitis patient and healthy control. We concluded that 10 types of these modifications (A_m, m6 2A, hm^6A, m^6G, m^7G, m^3C, m^5C, m^5C_m, hm^5C, Ψ) exhibited undetectable abundance, five modifications (m^3U, m^5U_m, m^1A, m^6A, and ac^4C) showed no

Table 2. The Signature Combination of Serum Modified Nucleosides Between the Subtypes of Uveitis

Subtypes	Idiopathic Uveitis	VKH	AS
Extremely Significant ($P < 0.001$)	C_m, m^5U, m^2G	m^2G, G_m, C_m, U_m	G_m, m^6A_m
Significant ($0.001 < P < 0.05$)	m^1G, $m^{2,2}G$, U_m, G_m	m^1G, m^6A_m	m^1G, m^2G
Not Significant ($P > 0.05$)	m^6A_m, m^3U, m^5U_m, m^1A, m^6A, ac^4C	m^5U, $m^{2,2}G$, m^3U, m^5U_m, m^1A, m^6A, ac^4C	$m^{2,2}G$, m^5U, U_m, C_m, m^3U, m^5U_m, m^1A, m^6A, ac^4C

significant differences between uveitis patients and healthy control, and the other eight modifications (m^2G, m^1G, m^6A_m, U_m, G_m, C_m, m^5U, and $m^{2,2}G$) displayed characteristic signature for various subtypes of uveitis. More importantly, our study suggests that the early precision diagnosis of uveitis can be developed on the basis of the signature combination of serum modified nucleosides. Although may be more time- and cost-efficient, the approach displayed high sensitivity and specificity, and suitable for precise and standardized examinations in clinical practice.

In a parallel to the post-transcriptional modifications of DNA and proteins, RNA molecules undergo over 170 distinct modifications,[47] These modifications, increasingly recognized as pivotal regulators in cell biology, have drawn analogies to the epigenetic landscape sculpted by DNA and histone alterations, leading to the coining of terms such as "RNA epigenetics"[48] and "epitranscriptome."[49,50] Over a dozen of modified nucleosides have demonstrated a crucial role in immunological responses in some inflammatory diseases like inflammatory bowel disease and rheumatoid arthritis.[51,52] For example, specific modified nucleosides like m^5U and m^6A can inhibit the activation of dendritic cells and the expression of toll-like receptors stimulated by RNA sensing.[19] These modifications exert their immunosuppressive effects by attenuating the secretion of pro-inflammatory cytokines, including TNF-α and IL-12, as well as modulating CD83 expression, thereby mitigating the inflammatory cascade.[19,53,54] Moreover, the m^6A modification has been implicated in influencing the innate immune response by modulating interferon signaling pathways.[55–57]

The immune and inflammatory responses is a key regulator for uveitis. For example, the significance of dendritic cells and toll-like receptors in the etiology of uveitis is highlighted by substantial evidence.[58–61] It has also been published that soluble CD83 can inhibit the actin-dependent calcium release in dendritic cells to alleviate uveitis.[62] The critical roles of TNF-α[11,63] and IFN[64] in ocular inflammation have been well documented, both experimentally and clinically, with anti-TNF-α therapies like adalimumab and infliximab gaining widespread clinical application.[63,65] Furthermore, IL-12 was reported to participate in regulating functions of a variety of effector cells, making IL-12 important therapeutic targets or agents in uveitis.[66] It is reasonable to assume that modified RNA plays a critical role by regulating the immune mediators like TNF-α, IFN, TLR, CD83, IL-12, and the activity of immune cells like dendritic cells in the development of uveitis. Supporting this, one study has reported that m^6A modification maintained sirtuin 1 mRNA stability in microglia, which reduced signal transducer and activator of transcription 3 phosphorylation, thus inhibiting microglial M1 polarization in uveitis[67]. However, until now, the role of RNA modification in uveitis is barely known.

This study elucidated that 8 modifications (m^2G, m^1G, m^6A_m, U_m, G_m, C_m, m^5U, and $m^{2,2}G$) displayed characteristic signature for various subtypes of uveitis, pointing to critical roles of these RNA modifications in disease pathogenesis. Nevertheless, it is acknowledged that the relatively small sample size may limit the generalizability of these findings and calls for larger cohorts to further validate subtype-specific nucleoside signatures. Additionally, this study focused on the diagnostic potential of modified nucleosides without exploring their mechanistic roles in uveitis pathogenesis. More studies are expected to elucidate the specific roles and mechanisms of these modified RNA in various uveitis diseases. Furthermore, certain differences are displayed depending on the ages and genders, more assays and larger cohorts are expected to determine the variations caused by ages and genders.

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