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VALIDATION OF THE NEWLY PROPOSED WHO CLASSIFICATION SYSTEM FOR CONJUNCTIVAL MELANOCYTIC INTRAEPITHELIAL LESIONS

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Response to Reviewers:	Point-by-Point Response, Revision 2

Re: AJO-20-1944R1

Manuscript:

"VALIDATION OF THE NEWLY PROPOSED WHO CLASSIFICATION SYSTEM FOR CONJUNCTIVAL MELANOCYTIC INTRAEPITHELIAL LESIONS"

Dear Dr. Parrish and reviewers,

Thank you for your helpful and constructive comments regarding our manuscript, "Validation of the newly proposed who classification system for conjunctival melanocytic intraepithelial lesions." We submitted this paper for publication in the Origina Article section of the American Journal of Ophthalmology. The information in our manuscript has not been published previously.
Each of the coauthors has seen and agrees with each of the changes made to this manuscript in the revision and to the way his or her name is listed.
The corresponding author had full access to all the data in the study and takes responsibility for the integrity of the data, the accuracy of the data analysis, and the decision to submit for publication.
We appreciate the opportunity to revise and improve our manuscript. Please find below our point-by-point response to the reviewers' and editorial comments.

Reviewer #1: The paper adds information to the literature plus a healthy bit of controversy. The primary author has done an admirable job at responding to a relatively long list of comments and recommendations.
Authors' response: We greatly appreciate the reviewer's constructive recommendations, which helped improve the manuscript and the reviewer's positive feedback!

Reviewer #2, Comment #1. The authors have responded to the comments and included explanations for the weaknesses of the study. One additional comment, the spelling of Lansing should be corrected.
Authors' response #1. We thank the reviewer for a thorough review and corrected the typographical error in the affiliations section of the Title page, as was recommended.

Reviewer 2 comment #2. Submitted Design section in the Abstract:
Design: Retrospective review and evaluation of classification systems.
Suggested Design section for the Abstract:
Design: Retrospective case series and evaluation of classification systems.
Authors' response #2. Thank you for the recommendation, which we incorporated into the revised Design section of the Abstract.

Editorial comments
Editorial comment #3. 3. Although your authorship citation is not incorrect, the AJO has recently updated its authorship policy, so you may wish to update how and where you have claimed dual authorship accordingly:
Shared positions – In line with our adherence to AMA guidelines, the AJO permits shared authorship between two authors for first and/or last author with the consent of the author group. First or last co-authorship can be shared between two authors if they have contributed equally according to ICMJE guidelines. The term "co-senior" is also permitted in lieu of "co-last." To designate shared authorship, mark the author names with an asterisk on the Title Page and state, "Dr X and Y contributed equally as co-first (or last) authors." This should also be noted in the Acknowledgement section. Only one Corresponding Author is permitted during the submission process, but a second Corresponding Author can be designated on the Title Page for post-publication communications. Other shared contributions will not be designated but their responsibilities should be delineated in the Author Contributions section of the Acknowledgement and CRediT statements.

Authors' response #3. We thank the journal for the opportunity to acknowledge equal contribution to the work of the co-first and co-senior authors. In accordance with the journal's guidelines, the title page states:
*Dr. Milman and Dr. Eiger-Moscovich contributed equally as co-first authors

	*Dr. Shields and Dr. Eagle contributed equally as co-senior authors We also added the following information to the Acknowledgment section: c. Other Acknowledgments: Dr. Milman and Dr. Eiger-Moscovich contributed equally as co-first authors; Dr. Shields and Dr. Eagle contributed equally as co-senior authors Thank you once again for your time in reviewing the manuscript and for the opportunity to revise and improve it. Respectfully, Tatyana Milman MD, Department of Pathology, Wills Eye Hospital 840 Walnut Street, Philadelphia, PA 19107. Tel: 215-928-3280 Fax: 215-825-4703 E-mail: tmilman@willseye.org

ABSTRACT

Purpose: To compare the sensitivity, specificity, accuracy, and inter-observer agreement of the two most commonly used classification systems for conjunctival melanocytic intraepithelial lesions with the new WHO classification.

Design: Retrospective case series and evaluation of classification systems.

Methods: Pathology and medical records of all patients, who underwent primary biopsy for conjunctival primary acquired melanosis (PAM) at Wills Eye Hospital between 1974 and 2002, with at least 36 months follow-up were reviewed. Data collected included age, sex, clinical findings, recurrence, and progression to melanoma. Twelve ophthalmic pathologists analyzed scanned hematoxylin-and-eosin stained virtual microscopic slides using three classification systems: PAM, conjunctival melanocytic intraepithelial neoplasia (C-MIN) and the World Health Organization (WHO) 4th edition classification of conjunctival melanocytic intraepithelial lesions (CMIL). Observer agreement, sensitivity, specificity, and diagnostic accuracy of each classification system were assessed.

Results: There were 64 patients who underwent 83 primary excisions with cryotherapy for conjunctival PAM, with adequate tissue for histopathologic evaluation. The inter-observer percent agreement in distinction between the low-grade and high-grade lesions was 76% for PAM, 67% for C-MIN, and 81% for WHO classification system. Low-grade lesions provided the greatest interpretative challenge with all three classification systems. The three classification systems had comparable accuracy of 81%-83% in their ability to identify lesions with potential for recurrence.

Conclusions: This study highlights the comparable strengths and limitations of the three classification systems for conjunctival melanocytic intraepithelial lesions and suggests that the simplified WHO classification scheme is appropriate for evaluation of these lesions.

October 21, 2020

Professor Richard K. Parrish II, MD
Editor-in-Chief
American Journal of Ophthalmology

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Point-by-Point Response, Revision 2

Re: AJO-20-1944R1

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Each of the coauthors has seen and agrees with each of the changes made to this manuscript in the revision and to the way his or her name is listed.

The corresponding author had full access to all the data in the study and takes responsibility for the integrity of the data, the accuracy of the data analysis, and the decision to submit for publication.

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Editorial comments

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Shared positions – In line with our adherence to AMA guidelines, the AJO permits shared authorship between two authors for first and/or last author with the consent of the author group. First or last co-authorship can be shared between two authors if they have contributed equally according to ICMJE guidelines. The term "co-senior" is also permitted in lieu of "co-last." To designate shared authorship, mark the author names with an asterisk on the Title Page and state, "Dr X and Y contributed equally as co-first (or last) authors." This should also be noted in the Acknowledgement section. Only one Corresponding Author is permitted during the submission process, but a second Corresponding Author can be designated on the Title Page for post-publication communications. Other shared contributions will not be designated but their responsibilities should be delineated in the Author Contributions section of the Acknowledgement and CRediT statements.

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***Dr. Milman and Dr. Eiger-Moscovich contributed equally as co-first authors**
***Dr. Shields and Dr. Eagle contributed equally as co-senior authors**

We also added the following information to the Acknowledgment section:

c. Other Acknowledgments: Dr. Milman and Dr. Eiger-Moscovich contributed equally as co-first authors**;** Dr. Shields and Dr. Eagle contributed equally as co-senior authors

Thank you once again for your time in reviewing the manuscript and for the opportunity to revise and improve it.

Respectfully,

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VALIDATION OF THE NEWLY PROPOSED WHO CLASSIFICATION SYSTEM FOR CONJUNCTIVAL MELANOCYTIC INTRAEPITHELIAL LESIONS: A COMPARISON WITH THE 'C-MIN' AND 'PAM' CLASSIFICATION SCHEMES

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Dr. Milman and Dr. Eiger-Moscovich contributed equally as co-first authors

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Short title: Conjunctival melanocytic intraepithelial lesions classification

Supplemental Material available at AJO.com

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INTRODUCTION

Conjunctival non-nevoid melanocytic lesions include benign, premalignant, and malignant conjunctival intraepithelial melanocytic proliferations and invasive melanoma.[1] Accurate diagnosis of conjunctival melanocytic intraepithelial lesions has significant prognostic and therapeutic implications. Although a subset of conjunctival melanocytic intraepithelial lesions follows a benign clinical course, some lesions progress to invasive conjunctival melanoma. This ocular surface cancer is a locally aggressive neoplasm with propensity for regional recurrence after treatment as high as 50%, and with the potential for metastasis and mortality of 20-30%.[2] The current gold standard for distinguishing between benign and premalignant or malignant conjunctival melanocytic intraepithelial proliferations is the histopathologic assessment of biopsied tissue.[3]

The terminology of conjunctival melanocytic intraepithelial lesions has been a matter of considerable debate over decades.[4] Historically, a site-specific terminology evolved to address the unique morbidity and management challenges of conjunctival melanocytic intraepithelial lesions.[3] Additionally, the site-specific histopathologic classification was prompted by the observation that most conjunctival melanocytic intraepithelial lesions could not be accurately subclassified using criteria associated with existing terminology derived from dermatopathology. As a result, the primary acquired melanosis (PAM) classification was developed by Folberg, McLean, and Zimmerman – a classification scheme that currently is widely used by both ophthalmologists and pathologists.[3] The PAM system was designed to replace two systems that led to over-treatment (Reese's precancerous melanosis that prompted orbital exenteration for benign conditions) or under-treatment (Zimmerman's "benign acquired melanosis" which led ophthalmologists to observe lesions with atypia until they developed into melanomas).[3] The term PAM is applied by clinicians to pigmented lesions of the conjunctiva that arise *de novo* and are not secondary to a known cause. What the ophthalmologist calls PAM clinically can range histopathologically from (1) deposition of melanin pigment within epithelial cells by normal-appearing melanocytes that are appropriately situated along the basal epithelium and are not increased in number (PAM without atypia); (2) proliferation of non-atypical melanocytes (PAM without atypia); (3) predominantly basilar hyperplasia of atypical non-epithelioid melanocytes (PAM with atypia, basilar pattern – frequently referred to as PAM with mild atypia); to (4) non-basilar hyperplasia of atypical melanocytes, including epithelioid melanocytes (PAM with atypia, other – frequently referred to as PAM with moderate or severe atypia).[3,5] This histopathologic classification scheme was based on the analysis of 41 cases of PAM with long-term outcome data and was documented to subdivide conjunctival melanocytic intraepithelial lesions into prognostically distinct categories.[3] Despite the widespread adoption of the PAM classification, several challenges emerged. These involve the inclusion of both epithelial pigmentation without melanocytic hyperplasia and melanocytic hyperplasia without atypia in the same category of PAM without atypia, the absence of melanoma *in situ* terminology, and the overall interpretative variability between the observers.[6-8]

In response to these challenges, Coupland and Damato developed the conjunctival melanocytic intraepithelial neoplasia (C-MIN) classification. The C-MIN classification adopted a scoring system to evaluate the radial spread (growth pattern), vertical spread (degree of intraepithelial involvement), and cytomorphology, with combined scores ranging from 1 to 10. The C-MIN classification proposed the term "conjunctival hypermelanosis" for non-proliferative conjunctival epithelial melanin pigmentation (C-MIN score 0), C-MIN with atypia (C-MIN scores 1-4), and suggested that the term *melanoma in situ* be applied to C-MIN scores 5 and greater.[6] Although this systematic histopathologic classification attempts to minimize inter-observer and intra-observer variability, observer concordance values have not been formally documented. Further, there are no formal outcome data to support stratification of C-MIN into the proposed categories. Additional concerns raised by experts include the implication that all conjunctival melanocytic intraepithelial proliferations are neoplastic, incorporation of vertical spread parameters into radial spread scoring, and that the methodology can be time-consuming.[7]

Neither the PAM nor the C-MIN classification system used terminology that is generally employed by surgical pathologists and dermatopathologists".

In January 2018, the consensus and editorial meeting for the 4th edition of the World Health Organization (WHO) Classification of Tumours of the Eye, met at the International Agency for Research into Cancer (IARC) in Lyon, France. During this meeting, at which 6 of the authors (RCE, SEC, HSM, HEG, CE, SH) were present, it was proposed that a simplified terminology be adopted that included the following stratification: (1) low-grade conjunctival melanocytic intraepithelial lesion (CMIL), corresponding to PAM without or with mild atypia and C-MIN scores 1-2; (2) high-grade CMIL, corresponding to PAM with moderate to severe atypia and C-MIN scores 3-5; and (3) melanoma in situ, corresponding to PAM with severe atypia involving >75% of the epithelium and a C-MIN score >5 **(Table 1).**[1] The WHO classification is relatively new and has not been formally validated for prognostic accuracy and observer variability.

We designed this study to assess the inter-observer variability, sensitivity, specificity, and accuracy of the recently developed WHO classification system for conjunctival melanocytic intraepithelial lesions by comparing it to the two most commonly used classification systems, PAM and C-MIN.

METHODS

The Wills Eye Hospital Institutional Review Board approved this study. The study was performed in compliance with HIPAA guidelines and with the tenets of Declaration of Helsinki.

Patient and tissue selection

We searched a dataset with long-term follow-up information[7] that originated from Wills Eye Hospital pathology and oncology records. We identified all consecutive patients who underwent primary surgery for conjunctival melanosis by the Wills Eye Hospital Ocular Oncology Service between 1974 and 2002. Melanosis of the conjunctiva was defined clinically as one or more patches of acquired asymmetric, flat, noncystic conjunctival and/or corneal brown pigmentation of at least 1 mm in diameter **(Figure 1).** All surgeries were performed with a standard technique,[9,10] and all patients had post-operative follow-up of at least 36 months. Histopathology slides were reviewed for adequacy. Patients with prior surgery, melanoma at presentation, a follow-up of less than 3 years, and those whose biopsy was suboptimal for histopathologic interpretation were excluded from analysis.

Data collected included patient age at the time of referral, sex, race, duration of symptoms, tumor laterality, anatomic location and size of the lesion, recurrence, progression to melanoma, and follow-up duration.

Histopathologic evaluation

Glass microslides stained with hematoxylin and eosin prepared from formalin-fixed paraffin-embedded tissues were scanned and digitized using an Aperio CS2 slide scanner (Leica Biosystems, Buffalo Grove, IL, USA) at 40x magnification and displayed with an Easyzoom viewer (Smart In Media GmbH & Co. KG, Köln, Germany; available at https://easyzoom.com/). Each digital slide was scored by 12 observers (TM, RCE, SEC, RF, HSM, HEG, CE, SH, CAH, MCHC, KL, SC) three times, once each using of the following systems: 1) primary acquired melanosis (PAM) as defined by Folberg et al.,[3] 2) conjunctival melanocytic intraepithelial neoplasm (C-MIN) as defined by Coupland et al.,[6] and 3) conjunctival melanocytic intraepithelial lesion (CMIL) as defined by the World Health Organization (WHO)[1] **(Figure 1, Table 1).** The WHO classification was designed to "bridge the terminology gap" between the PAM and C-MIN classification systems and did not provide WHO classification-specific morphologic criteria. Each author classified CMIL lesions based on his or her individual interpretation of the WHO classification. The observers were masked to all clinical data with the exception of patient age. Scoring by each of the 3 systems was performed by each observer on

average 16 days apart (median 10, range 0-56). The classification system order, with which each observer scored the slides was recorded. Additional parameters assessed were 1) the classification system(s) used in pathology practice by each observer, 2) length of time required to establish the diagnosis with each specific classification system for each observer, and 3) the assessment of conjunctival resection margin status (performed by 2 observers, TM and RCE).

Statistical analysis

Summary statistics are reported for demographic, clinical, and pathological characteristics on a patient and biopsy level. The clinical data at the time of surgery were used for patient-level analyses. In biopsy-level comparisons, inter-rater agreement was tested by Fleiss Kappa when comparing 3 or more raters and Cohen's Kappa when comparing 2 raters. For the PAM classification system, 11 observers were compared to Dr. Robert Folberg, whose scores were used as "PAM benchmark standard" based on his contribution to the PAM classification system and his experience with this system. For the C-MIN classification system, 11 observers were compared to Dr. Sarah Coupland, based on her contribution to the C-MIN classification system and her experience with this system. Because the WHO classification system was proposed by a panel of experts (which included 6 of 12 observers) without a defined "benchmark standard", concordance values were calculated for all 12 observers. Diagnostic test statistics including sensitivity and specificity for the PAM, C-MIN, and WHO classification systems were calculated for each observer. In order to assess diagnostic accuracy, empirical receiver operating characteristic (ROC) curves were constructed for each observer and the areas under the ROC curves (AUROC) were calculated. The PROC LOGISTIC procedure within SAS was used to calculate sensitivity, specificity, and probability for each threshold value, and to plot an ROC curve an AUC estimate, and significance level (compared to the diagonal curve). For each classification system, an average ROC plot was calculated by averaging the sensitivity and specificity values at each threshold, as detailed by Chen et al.[10] Comparative analysis of time needed for interpretation was performed via Friedman test for 3 or more comparisons followed by post hoc analysis with Wilcoxon Rank Sum tests. Kappa statistics were calculated in SPSS (version 26). All other analyses were performed in SAS V9.4 (SAS Institute Inc., Cary, NC). Two-sided $P<.05$ was considered to be statistically significant.

RESULTS

Patient demographics and clinical characteristics

Patient demographics and clinical characteristics are summarized in **Table 2** and documented in **Figure 1**. Sixty-four patients (64 eyes), who underwent 83 biopsies were included in this analysis. Mean patient age at the time of referral was 56 years (median 56, range, 15-89), 46 (72%) were female, and 18 (28%) were male. The race was recorded as white in 57 (89%), Hispanic in 5 (8%), and black in 2 (3%). Anatomic locations involved were bulbar conjunctiva 59 (92%), limbal conjunctiva 44 (69%), cornea 27 (42%), forniceal conjunctiva 10 (16%), tarsal conjunctiva 7 (11%), and caruncle/plica semilunaris 9 (14%). Melanosis involved a mean of 2 clock hours of conjunctiva (median 1, range 1-3 clock hours) with a mean largest dimension of 10 mm (median 8, range 1-40). All patients underwent excisional biopsy combined with cryotherapy using a no touch technique.[8,9] The median follow-up was 36 months (mean 77, range 36-349). Thirteen patients (20%) experienced recurrence. The mean time to recurrence was 20 months (median 20, range 6-35). Two (3%) recurrent lesions progressed to melanoma, 62 months and 207 months following initial surgery, respectively.

Histopathology

Time required for biopsy interpretation

Eighty-three biopsies were reviewed independently by 12 observers. In routine pathology practice all observers used the PAM classification system, 7 (58%) observers used the C-MIN classification system, and 4 (33%) observers used the WHO classification system. Ten (83%) observers used more than one classification system, most commonly PAM and C-MIN (7/12, 58%) **(Table 3).** The time needed for interpretation of a biopsy by all observers was longer for the C-MIN classification system (mean 1.8 minutes, standard deviation [SD] 1.2, median 1.5, range <0.1-9.1) when compared to the PAM (mean 1.7 minutes, SD 1.2, median 1.4, range 0.1-11) and WHO (mean 1.5 minutes, SD 1.1, median 1.1, range <0.1-11) classification systems (*P* <.001) **(Table 3).** A subgroup analysis demonstrated that pathologists using the C-MIN system in routine practice spend significantly more time for interpretation than those who do not (median 2.0 vs. 1.2 minutes, *P*<.001) while those routinely using the WHO system spend significantly less time for interpretation than those who do not (median 1.0 versus 1.3 minutes, *P*=.006)

Agreement between observers

The agreement between each observer and the benchmark interpretation by Dr. Robert Folberg for the PAM classification system is summarized in the **Supplemental Table 1** (Supplemental Material at AJO.com). The agreement between each observer and the benchmark interpretation by Dr. Sarah Coupland for the C-MIN classification system is summarized in the **Supplemental Table 2** (Supplemental Material at AJO.com). The agreement between all 12 observers collectively for the PAM, C-MIN, and WHO classification systems is summarized in the **Table 4.** The conjunctival melanocytic intraepithelial lesions with excellent concordance between all observers are depicted in **Figure 1***.* The percent agreement between observers was calculated as a number of agreement scores divided by the total number of scores x100. Fleiss Kappa values were interpreted as described by Landis and Koch (< 0 Poor agreement, 0.01 – 0.20 Slight agreement, 0.21 – 0.40 Fair agreement, 0.41 – 0.60 Moderate agreement, 0.61 – 0.80 Substantial agreement, 0.81 – 1.00 Almost perfect agreement).[12]

PAM classification system: The concordance with the benchmark standard for individual PAM groupings (0 vs. 1 vs. 2) was fair and ranged from 0.21 to 0.47 for an individual observer, with an overall mean concordance between 11 observers and the benchmark standard of 0.34 (95% confidence interval [CI] 0.28-0.40) (**Supplemental Table 1**, Supplemental Material at AJO.com). Analysis of PAM groupings showed that separating PAM into 0-1 vs. 2 categories improved the overall mean agreement with the benchmark standard to moderate (0.53, CI 0.44-0.61) with 5 (45%) observers having a substantial agreement (0.60-0.70) (**Supplemental Table 1,** Supplemental Material at AJO.com). The distinction of PAM without atypia (score 0) and PAM with atypia, basilar hyperplasia (score 1) presented the greatest interpretative challenge. Ten (91%) observers assigned a lower score “0” when compared to the benchmark standard. Additional difficulty was in interpretation of a pagetoid spread of melanocytes that lacked overt cytomorphologic atypia. Representative examples of lesions that provided greatest interpretative challenge are depicted in **Figure 2**.

The agreement between all 12 observers collectively for individual PAM groupings (0 vs. 1 vs. 2) was fair (Fleiss Kappa 0.37, CI 0.35-0.39, *P*<.0001), 55% agreement. The agreement improved to moderate with PAM (0-1 vs. 2) grouping (Fleiss Kappa 0.58, CI 0.55-0.61, *P*<.0001), 76% agreement **(Table 4).**

C-MIN classification system: We selected C-MIN groupings (0-4 vs. 5-10) and (0-5 vs. 6-10) based on the premise that these groupings accurately delineate between prognostically favorable and unfavorable intraepithelial melanocytic proliferations.[1,6] The individual observer concordance with the benchmark standard for C-MIN (0-4 vs. 5-10) and C-MIN (0-5 vs. 6-10) groupings ranged from fair-to-substantial (0.24-0.81), with an overall mean substantial concordance between 11 observers and the benchmark standard for C-MIN (0-4 vs. 5-10) (Kappa 0.64, CI 0.56-0.71) and C-MIN (0-5 vs. 6-10) (Kappa 0.61, CI 0.51-0.71) **Supplemental Table 2** (Supplemental Material at AJO.com). The mean concordance of observers who use C-

MIN classification system in routine practice was slightly but not significantly higher, when compared to observers who do not use C-MIN classification system in routine practice (0.67 vs. 0.60, *P* = .33 for C-MIN 0-4 vs. 5-10 and 0.66 vs. 0.57, *P* = .43 for C-MIN 0-5 vs. 6-10) (**Supplemental Table 2**, Supplemental Material at AJO.com).

Analysis of C-MIN sub-groupings showed a fair concordance in the interpretation of radial spread (0.26, CI 0.20-0.33), vertical spread (0.31, CI 0.21-0.41), and cytomorphologic melanocyte atypia (0.26, CI 0.18-0.35) (**Supplemental Table 2**, Supplemental Material at AJO.com). The distinction of the "Nil" growth pattern from "pagetoid spread" presented the greatest interpretative challenge. When compared with the benchmark standard, 9 (82%) observers were more likely to interpret the growth pattern as "pagetoid spread", when compared to the more frequent "Nil" interpretation by the benchmark standard. Interpretation of vertical spread was overall extremely heterogeneous. However, there was a trend by the observers (7/11, 64%) to more frequently interpret the vertical spread as "<50%", when compared to the more frequent "Nil" interpretation by the benchmark standard**.** Assessment of melanocyte nuclear size with respect to the basal epithelial cell presented the greatest challenge of cytomorphologic analysis. All observers interpreted nuclear enlargement in melanocytes with greater frequency when compared to the more frequent "normal" (nucleus/cytoplasm < basal epithelial cell) interpretation by the benchmark standard**.** Representative examples of lesions that provided greatest interpretative challenge are depicted in **Figure 2**.

The agreement between all 12 observers collectively for C-MIN (0-4 vs. 5-10) grouping and C-MIN (0-5 vs. 6-10) grouping was moderate at Fleiss Kappa 0.52 [CI 0.49-0.56, *P*<.0001 for C-MIN (0-4 vs. 5-10) and CI 0.48-0.55, *P*<.0001 for C-MIN (0-5 vs. 6-10)], 67% agreement **(Table 4).**

WHO classification system: The WHO classification system was proposed by an expert panel of ophthalmic pathologists with no defined benchmark standard. The agreement between all 12 observers collectively for individual WHO groupings (0 vs. 1 vs. 2 vs. 3) was fair (Fleiss Kappa 0.26, CI 0.24-0.28, *P*<.0001), 46% agreement. The agreement improved to fair-to-moderate with WHO (0-1 vs. 2-3) and WHO (0-2 vs. 3) groupings at Fleiss Kappa 0.36 [CI 0.34-0.38, *P*<.0001 for WHO (0-1 vs. 2-3) and CI 0.35-0.36, *P*<.0001 for WHO (0-2 vs. 3)] **(Table 4).** The absolute percent agreement for WHO (0-1 vs. 2-3) and WHO (0-2 vs.3) scoring was 81% and 85%, respectively. The low variability between the WHO scores, when compared with PAM and C-MIN scores, accounted for the lower Fleiss kappa reliability values. Representative examples of lesions that provided greatest interpretative challenge are depicted in **Figure 2**.

Sensitivity, specificity, and accuracy

Because only 2 lesions progressed to invasive melanoma, we chose recurrence as an indicator of aggressive behavior. The sensitivity, specificity, and accuracy analyses were performed on the highest-grade biopsy (when multiple sites were biopsied) from each of 64 patients (total 64 biopsies).

The PAM classification system had an average accuracy (as estimated by the area under receiver operating characteristic curve, AUC) of 81% (SD 5). The diagnosis of PAM with any degree of atypia (0 vs. 1-2 grouping) had a mean sensitivity of 95% (SD 4) and mean specificity of 38% (SD 18). The diagnosis of PAM with cytomorphologic atypia and non-basilar spread or epithelioid morphology (0-1 vs. 2 grouping) yielded the most optimal combined sensitivity (86%, SD 10) and specificity (75%, SD 11) **(Table 5, Figure 3).**

The C-MIN classification system had an average accuracy of 83% (SD 4). The diagnosis of C-MIN 3 or higher (0-2 vs. 3-10 grouping) had a mean sensitivity of 92% (SD 3) and mean specificity of 53% (SD 24). The diagnosis of melanoma in situ (0-4 vs. 5-10) grouping yielded the most optimal combination of sensitivity (83%, SD 11) and specificity (73%, SD 16) **(Table 5, Figure 3).**

The WHO classification system had an average accuracy of 83% (SD 6). The diagnosis of low-grade CMIL or higher (0 vs. 1-3 grouping) had a mean sensitivity of 97% (SD 4) and mean

specificity of 30% (SD 19). The diagnosis of high-grade CMIL or melanoma in situ (0-1 vs. 2-3 grouping) yielded the most optimal combined sensitivity (81%, SD 27) and specificity (76%, SD 6) **(Table 5, Figure 3).** A subgroup analysis revealed no significant difference in the average accuracy of observers who use WHO classification routinely versus those who do not (85% [SD 9], range 71-92%, median 88% vs. 82% [SD 5], range 72-85%, median 83.0%; *P*=0.19).

Influence of classification system scoring order on observer accuracy

Multivariate analysis of the order in which pathologists performed each set of ratings, controlling for the type of classification system, revealed no significant "learning effect". The first, second, and third order of scoring had the mean accuracies of 81%, 79%, 85% respectively (*P*=0.28)

Analysis of lesions that recurred

The analysis of 13 conjunctival biopsies from patients who later developed recurrence showed that with PAM and the C-MIN classification by the benchmark standards, 12 of 13 lesions were classified as high-grade atypia / melanoma in situ (**Table 6**). Two lesions that were classified as high-grade atypia / melanoma in situ by all 12 observers progressed to invasive melanoma (Patients 11 and 12, **Table 6**). One lesion in a 33-year-old Hispanic female was interpreted predominantly by observers as either conjunctival hypermelanosis or PAM/ C-MIN/ CMIL with low-grade atypia (Patient 13, **Table 6, Figure 2D**). In this patient recurrence was noted 10 months post-operatively and the patient was observed without biopsy with stable disease.

Assessment of margin status

All conjunctival biopsies that featured intraepithelial melanocyte hyperplasia with or without accompanying atypia contained hyperplastic and/or atypical melanocytes at the excision margins.

DISCUSSION

Conjunctival melanoma is a neoplasm with high propensity for regional recurrence, locally aggressive behavior, and metastasis.[2,13,14] Conjunctival melanoma arising from PAM/conjunctival melanocytic intraepithelial lesion has been documented to have 10-year metastasis and mortality rates of 25% and 9%, respectively.[13] The reported rate of progression of PAM to invasive melanoma following treatment ranges from 13% to 46%.[9,15] The distinction between benign, low-risk and high-risk pre-malignant conjunctival melanocytic intraepithelial lesions is made on histopathologic evaluation of biopsied tissue, underscoring the importance of accurate histopathologic diagnosis.[3] The ideal classification system should be (1) sensitive, specific, and accurate; (2) easy to use in routine pathology practice with low inter-observer and intra-observer variability; and (3) clinically relevant and readily adaptable to clinical practice (and reflect the biology of the lesion).

In this comparative analysis of the three most commonly used classification systems (PAM, C-MIN, and WHO), we noted comparable accuracy in the ability of all three systems to identify the lesions with a potential for recurrence. In all three classification systems, the agreement between observers was highest when discriminating between the low-grade and high-grade lesions. The percent agreement between the observers in distinction between PAM score 0-1, C-MIN score 0-4, WHO score 0-1 versus PAM score 2, C-MIN score 5-10, and WHO score 2-3 was 76%, 67%, and 81%, respectively. Compared to the PAM and WHO classification systems, C-MIN classification took slightly longer.

Systematic analysis of lesions that were highly discordant between the observers highlighted limitations of current classification schemes. Most interpretative challenges lay in analysis of low-grade lesions: (1) the separation of non-proliferative conjunctival epithelial hypermelanosis from conjunctival melanocytic intraepithelial proliferation of cytomorphologically normal or minimally atypical melanocytes, and (2) in the interpretation of pagetoid intraepithelial spread of cytomorphologically normal or minimally atypical melanocytes. Theoretically, these interpretative challenges can be, in part, overcome with the use of ancillary

immunohistochemical stains (such as Melan-A, SOX10, and S100P) to assess the number and distribution of melanocytes and to highlight subtle nuclear and cytoplasmic alteration. Although all three classification systems were developed based strictly on morphologic parameters, a method reproduced in our study, in these challenging cases 5 of 12 (42%) observers suggested that immunohistochemical stains may improve diagnostic accuracy and also facilitate assessment. In our study, only a subset of tissues had sufficient material for ancillary immunohistochemical studies, which in many cases did not contain an area of interest that was present in the initial hematoxylin and eosin stained section. These limitations precluded conclusive assessment of the effect of immunohistochemical stains on the sensitivity, specificity, accuracy, and observer variability of each classification and suggest an area of interest for future studies. Of note, in a recent retrospective study of 47 conjunctival biopsies we evaluated the usefulness of Melan-A, SOX-10, HMB45, and p16 immunohistochemical stains in the distinction between the low-grade and high-grade conjunctival melanocytic intraepithelial lesions.[16] We found that although the stains for melanocytic markers Melan-A and SOX-10 facilitate assessment of melanocytic intraepithelial lesions, the current immunohistochemical panels have limited value in distinction between the low-grade and high-grade intraepithelial melanocytic lesions and need to be used judiciously.[16]

In this study we attempted to minimize bias by masking the observers to all clinical data with the exception of patient age. However, in reality, clinical information (race, disease extent, disease progression) greatly influence the histopathologic diagnosis, particularly in challenging cases that feature mild melanocyte hyperplasia without overt cytomorphologic atypia. It is possible that the availability of complete clinical data will enhance both the concordance between the observers and the accuracy of the current classification schemes.

Our study was designed to overcome some of the challenges that prevented similar analyses by other investigators. We selected only primary biopsies from patients with at least 36-month follow-up managed uniformly at a single oncology center. We created virtual digital histopathology slides from the glass microslides, ensuring that all observers reviewed the same material. Finally, we collected a group of 12 ophthalmic pathologists, which included the originators of all 3 classification schemes, thus providing a benchmark standard for assessment.

The stringent inclusion and exclusion criteria in this study limited the sample size, and small sample size is one important limitation of our analysis. Since there were too few progressions to melanoma to use as the primary clinical outcome, we selected recurrence following biopsy with cryotherapy as an outcome. Recurrence as an outcome is another significant limitation of this study. It is important to note that while we attempted to include in our study the patients with uniform primary treatment, the subsequent care was rendered dependent on histopathologic diagnosis, contributing to the outcome bias. Additionally, although the mean time to recurrence of PAM following excision and cryotherapy has been estimated at 19 months and the mean time to recurrence of PAM with development of melanoma has been estimated at 30 to 39 months, late recurrences can occur.[3,9] Our patient cohort had a median follow-up time of 36 months, which may have underestimated the recurrence and progression to melanoma, limiting our assessment of the diagnostic accuracy of each classification. We also acknowledge the likelihood of bias introduced by excluding the outcome data of the patients with follow-up of less than 36 months.

As mentioned previously, the retrospective design with assessment of archival tissue made our analysis of the impact of immunohistochemical stains on diagnostic accuracy and concordance inconclusive. Further, although we assessed the inter-observer reproducibility of three classification systems, we did not assess intra-observer reproducibility, a limitation that stems from the inherent challenges of conducting this assessment by a group of 12 observers. We attempted to minimize the observer “learning curve” by temporally separating scoring with each classification system and attempted to minimize bias by allowing each observer to decide in what order to conduct the scoring. However, to completely eliminate bias introduced by repetitive scoring of microslides one would need to create separate datasets, which would reduce the power of our study. Finally, although we attempted to minimize observer bias by creating virtual digital histopathology slides, this mode of assessment does not identically

replicate the experience with a microscope and creates artifacts leading to interpretative challenges. It is also important to note that although for simplification of methodology we limited our comparison of the WHO classification system to the two most commonly used classification schemes, other classification systems exist, such as the intraepithelial melanocytic proliferation (IMP) classification developed by Frederick Jakobiec and melanocytic conjunctival lesions (MCL) scoring system developed by Alexander Maly et al.[7,17]

Despite these limitations, we believe that our study provides a much-needed comparative analysis of the three classifications systems, which appear to have comparable sensitivity, specificity, and accuracy. Based on these data, it is reasonable for pathologists to use WHO classification system when generating reports. The WHO classification system has high sensitivity of 97% and specificity of 30% for detection of low-grade CMIL (a good screening cut-off), a sensitivity of 81% and specificity of 76% for detection of high-grade CMIL, and an accuracy of 83%. Thus, WHO classification system provides a good delineation between the lesions with no potential for recurrence (conjunctival epithelial hypermelanosis), low potential for recurrence (low-grade CMIL), and a high potential for recurrence (high-grade CMIL or melanoma in situ). Evaluation with WHO classification system takes no longer than with PAM classification. The WHO classification system terminology is readily generalizable to non-ophthalmic pathology practice. Further, the WHO nomenclature translates PAM and C-MIN to terms non-pathologists and non-ophthalmologists might understand, emerging as a “Rosetta stone” for moving between the PAM and C-MIN systems.

We should be clear that the current WHO classification does not provide explicit histologic criteria for CMIL classification. Our evaluation offers an opportunity to provide histologic criteria for CMIL classification and suggests that simplification of WHO classification into a binary low-grade and high-grade system, as summarized in the **Table 7**, will yield both high inter-observer concordance and accurately reflect the biology of conjunctival melanocytic intraepithelial lesions. This provisional simplified WHO classification will be formally validated by our group. The inter-observer concordance and the diagnostic accuracy of any of the existing systems, including the WHO classification system, could be further enhanced by providing a detailed monograph accompanied by histopathologic atlases and educational courses. A formal integration of immunohistochemical stains into the classification scheme may also serve to minimize the interpretative challenges.

Although the WHO classification system is appropriate in the pathology context, separation of conjunctival epithelial acquired pigmentation into primary, skin-tone associated, and secondary, as outlined in the PAM classification remains appropriate and pertinent in a clinical context. Ultimately, with the refinement of digital pathology and implementation of artificial intelligence and deep learning algorithms already being applied in uveal melanomas,[18] as well with the refinement of our molecular genetic methods,[19-21] an integrated morphologic-molecular classification will emerge that will further improve our ability to accurately diagnose these challenging lesions.

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Data collection: MEM, TM, RCE, CLS
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LEGENDS

Figure 1. Clinical characteristics of conjunctival melanocytic intraepithelial lesions with corresponding histopathology that was highly concordant between observers. Histopathology images are taken from virtual slides distributed to all observers.
A. Patchy pigmentation involves less than 1 clock hour of the limbal and bulbar conjunctiva.
B. Corresponding histopathology shows normally distributed small melanocytes along the basal epithelium (arrow) with pigment in the epithelial cells, compatible with PAM without atypia or conjunctival hypermelanosis (C-MIN 0, CMIL 0) [hematoxylin-eosin stain, 35x].
C. Patchy pigmentation involves 1-2 clocks hour of the limbal and bulbar conjunctiva.
D. Corresponding histopathology shows hyperplasia of basally distributed melanocytes with mildly enlarged hyperchromatic nuclei (arrow), compatible with PAM 1 (basilar hyperplasia/ mild atypia), C-MIN 2, low-grade CMIL [hematoxylin-eosin stain, 35x].
E. Patchy pigmentation involves 3 clock hours of the limbal and bulbar conjunctiva and extends into the adjacent cornea.
F. Corresponding histopathology shows proliferation of small epithelioid melanocytes with enlarged nuclei and expanded cytoplasm, arranged in nests (arrow), with intraepithelial migration, compatible with PAM with severe atypia, C-MIN 7 / melanoma in situ [hematoxylin-eosin stain, 35x].
G. Patchy pigmentation involves the plica semilunaris and the adjacent bulbar and forniceal conjunctiva. This lesion recurred.
H. Corresponding histopathology shows proliferation of enlarged, polyhedral, dendritic melanocytes with intraepithelial migration and prominent pagetoid scatter (arrow), compatible with PAM with severe atypia, C-MIN 6 / melanoma in situ [hematoxylin-eosin stain, 35x].

Figure 2. Examples of cases with highly discordant interpretation by observers. Histopathology images are taken from virtual slides distributed to all observers.
A. This lesion features a cellular epithelium that contains rare cells with small hyperchromatic nuclei (arrows) in superficial layers, raising concern for intraepithelial melanocytic proliferation. The diagnoses varied from PAM without atypia and C-MIN 0/ CMIL 0 to PAM with mild atypia, C-MIN 1-4, or low-grade CMIL [hematoxylin-eosin stain, 35x].
B. This lesion has a cytoplasmic clearing artifact around the nuclei of epithelial cells (arrow), making assessment of melanocytic component challenging. The diagnoses varied from PAM without atypia and C-MIN 0 to PAM with severe atypia and melanoma in situ [hematoxylin-eosin stain, 35x].
C. Interpretation of this lesion was complicated by slightly eccentric sectioning and challenge in distinguishing basal epithelial cells from a small focus of mildly enlarged atypical melanocytes (arrow). Interpretations ranged from PAM without atypia and C-MIN 0 to PAM with severe atypia and melanoma in situ [hematoxylin-eosin stain, 35x].
D. This lesion features a small focus of enlarged polyhedral melanocytes (arrows). SOX-10 immunohistochemical stain provided for observers (not shown) showed basally distributed and normal in density melanocytes. Most observers favored either conjunctival hypermelanosis or PAM without atypia. The lesion recurred [hematoxylin-eosin stain, 35x].
E. A small focus of hyperplastic mildly enlarged melanocytes in an otherwise unremarkable biopsy. Interpretations ranged from conjunctival hypermelanosis /PAM without atypia to PAM with severe atypia / high-grade C-MIN / high-grade C-MIL [hematoxylin-eosin stain, 35x].
F. This lesion features a prominent intraepithelial pagetoid scatter of melanocytes that lack overt cytomorphologic atypia. The observer interpretations ranged from PAM without atypia and low-grade C-MIN 0-2/ low-grade CMIL to PAM with severe atypia / melanoma in situ [hematoxylin-eosin stain, 35x].

Figure 3. An average receiver operating characteristic (ROC) curve (black line) was constructed for PAM classification, C-MIN classification, and WHO classification by taking the mean

sensitivity and specificity values at each threshold for each of the 12 raters. The upper and lower boundaries are the standard deviation (grey lines). The y-axis (Sensitivity) denotes the true positive rate (recurrence of the lesions scored as non-“0” with 3 classifications: PAM 1-2, C-MIN 1-10, WHO 1-3). The x-axis (1-Specificity) denotes the false positive rate (absence of recurrence of the lesions scored non-“0” with 3 classifications: PAM 1-2, C-MIN 1-10, and WHO 1-3). All 3 classifications systems have comparable area under receiver operating characteristic curve (AUC).

Table 1

Table 1. Conjunctival melanocytic intraepithelial lesions: PAM, C-MIN, and WHO classification*

PAM system modified to include melanoma in situ	PAM scoring in this study	C-MIN scoring system (0-10)	Proposed WHO classification system	WHO scoring in this study
PAM without atypia (hyperpigmentation only)	PAM score = 0	Conjunctival hypermelanosis C-MIN score = 0	Hyperpigmentation only	WHO score = 0
PAM without atypia (melanocytic hyperplasia without atypia) PAM with mild atypia	PAM score = 0 PAM score = 1	C-MIN score = 1 C-MIN score = 2	Low-grade CMIL	WHO score = 1
PAM with moderate atypia (no epithelioid cells) PAM with severe atypia (epithelioid cells but not full thickness)	PAM score = 2	C-MIN score = 3 C-MIN score = 4-5	High-grade CMIL	WHO score = 2
Melanoma in situ	PAM score = 2	C-MIN score >5	Melanoma in situ	WHO score = 3

* Adapted from WHO, 4th edition classification of conjunctival intraepithelial melanocytic lesions.[1]

PAM = primary acquired melanosis, C-MIN = conjunctival melanocytic intraepithelial neoplasia, WHO = World Health Organization, CMIL = conjunctival melanocytic intraepithelial lesion

PAM classification key (scored from 0 to 2 in this study)[3]

Score 0: PAM without atypia = increased pigment in epithelial cells without melanocytic hyperplasia or atypia, melanocytic hyperplasia without cytomorphologic atypia

Score 1: PAM with cytomorphologic atypia (enlarged dendritic or polyhedral melanocytes), basilar hyperplasia pattern [PAM with mild atypia]

Score 2: PAM with cytomorphologic atypia, other (non-basilar hyperplasia and/or epithelioid morphology) [PAM with moderate / severe atypia]

C-MIN scoring system is scored out of 10 (cumulative score from 0 to 10 in this study)[6]

Score 0-4: *Growth pattern /radial (horizontal) spread*

Nil (score 0), basal (score 1), pagetoid (score 2), isolated nests (score 3), confluent nests (score 4)

Score 0-3: *Vertical spread (suprabasilar extension)*

Nil (score 0), 1-50% (score 1), 50-90% (score 2), >90% (score 3)

Scored 0-3: *Melanocytic atypia (cumulative score 0-3)*

Nucleus/cytoplasm <basal squamous cell (score 0), nucleus $\geq$ basal squamous cell (score 1), cytoplasm > basal squamous cell (score 1), Nucleoli and/or mitoses (score 1)

WHO classification key (scored from 0 to 3 in this study)

Score 0: Conjunctival epithelial hypermelanosis = Increased pigment in epithelial cells without melanocytic hyperplasia or atypia

Score 1: Low-grade CMIL = Predominantly basilar melanocytic hyperplasia without atypia or with mild atypia (dendritic or small polyhedral, non-epithelioid melanocytes)

Score 2: High-grade CMIL = Significant non-basilar melanocytic hyperplasia involving <50% of epithelium without nests and/or melanocytes with epithelioid morphology

Score 3: Melanoma in situ = Atypical melanocytes with nesting and/or >50% full-thickness intraepithelial migration

Table 2

Table 2. Patient demographics and clinical characteristics	
Variable	**Value**
Age (years)	
Mean (SD), median (min-max)	56 (17), 56 (15-89)
Race	
Caucasian	57 (89%)
Hispanic	5 (8%)
Black	2 (3%)
Sex	
Male	18 (28%)
Female	46 (72%)
Involved eye	
OD	32 (50%)
OS	32 (50%)
Symptoms	
None	24 (38%)
Pigmented spot/discoloration	39 (61%)
Erythema	1 (1%)
Duration of symptoms before surgery (months)	
Mean (SD), median (min-max)	59 (65), 33 (0-245)
Growth	
No growth	23 (59%)
Growth	16 (41%)
Size (mm)	
Mean (SD), median (min-max)	10 (8), 8 (1-40)
Clock hours	
Mean (SD), median (min-max)	1 (0.5), 1 (1-3)
Focality	
Unifocal	43 (67%)
Multifocal	21 (33%)
Location	
Bulbar conjunctiva	59 (92%)
Limbus	44 (69%)
Cornea	27 (42%)
Fornix	10 (16%)
Palpebral conjunctiva	7 (11%)
Caruncle/Plica	9 (14%)
Eyelid margin/skin	1 (1%)
Number of sites biopsied in each patient	
1	50 (78%)
2	10 (16%)
3	4 (6%)
Follow-up duration (months)	
Mean (SD), median (min-max)	77 (69), 36 (36-349)
Recurrence	
Yes	13 (22%)
No	47 (78%)
Time from treatment to recurrence (months)	
Mean (SD), median (min-max)	20 (10), 20 (6-35)
Progression to invasive malignant melanoma	
Yes	2 (3%)
No	62 (97%)

SD = standard deviation, min = minimum, max = maximum

Table 3

Table 3. Time (minutes) needed for interpretation of a biopsy by each classification system: mean (SD), mean 95% CI, median (min, max). Gray box denotes a system used in routine practice

Obs. #	PAM	C-MIN	WHO
1	Mean 1.0 (0.9), CI (0.8, 1.2) Median 0.7 (0.2, 5.2)	Mean 1.3 (1.1), CI (1.0, 1.5) Median 0.9 (0.2, 5.5)	Mean 0.9 (0.8), CI (0.8, 1.1) Median 0.7 (0.1, 4.1)
2	Mean 1.7 (1.0), CI (1.5, 2.0) Median 1.6 (0.2, 4.7)	Mean 1.9 (1.2), CI (1.6, 2.1) Median 1.5 (0.2, 5.0)	Mean 1.0 (0.7), CI (0.8, 1.1) Median 0.8 (<0.1, 3.3)
3	Mean 0.3 (0.1), CI (0.3, 0.3) Median 0.3 (0.2, 0.8)	Mean 0.6 (0.3), CI (0.5, 0.6) Median 0.6 (<0.1, 1.0)	Mean 0.3 (0.1), CI (0.3, 0.3) Median 0.3 (0.2, 0.8)
4	Mean 0.6 (0.3), CI (0.5, 0.7) Median 0.5 (<0.1, 1.6)	Mean 0.8 (0.4), CI (0.7, 0.9) Median 0.8 (0.3, 2.3)	Mean 0.8 (0.4), CI (0.7, 0.9) Median 0.7 (0.3, 2.3)
5	Mean 1.5 (0.8), CI (1.3, 1.6) Median 1.1 (0.7, 4.2)	Mean 1.6 (0.9), CI (1.4, 1.9) Median 1.3 (0.7, 5.0)	Mean 1.4 (0.7), CI (1.2, 1.5) Median 1.1 (0.7, 4.0)
6	Mean 2.3 (1.6), CI (2.0, 2.6) Median 2.0 (0.3, 11)	Mean 2.5 (1.4), CI (2.2, 2.8) Median 2.3 (0.1, 7.8)	Mean 2.4 (1.7), CI (2.0, 2.8) Median 2.0 (0.3, 11)
7	Mean 2.1 (1.1), CI (1.8, 2.3) Median 2.0 (0.3, 4.8)	Mean 2.1 (1.4), CI (1.8, 2.4) Median 1.8 (0.5, 7.4)	Mean 1.7 (1.0), CI (1.5, 1.9) Median 1.6 (0.3, 4.9)
8	Mean 2.7 (1.0), CI (2.5, 2.9) Median 3.0 (1.0, 7.0)	Mean 1.8 (0.7), CI (1.7, 2.0) Median 2.0 (1.0, 4.0)	Mean 1.8 (0.7), CI (1.6, 2.0) Median 2.0 (1.0, 4.0)
9	Mean 1.6 (1.0), CI (1.4, 1.9) Median 1.5 (0.1, 4.7)	Mean 3.1 (1.4), CI (2.8, 3.4) Median 3.0 (0.1, 9.1)	Mean 1.5 (0.8), CI (1.3, 1.6) Median 1.3 (0.3, 4.3)
10	Mean 2.1 (0.8), CI (2.0, 2.3) Median 2.0 (1.0, 5.0)	Mean 2.1 (0.8), CI (1.9, 2.3) Median 2.0 (1.0, 4.0)	Mean 2.8 (0.9), CI (2.6, 3.0) Median 3.0 (1.0, 5.0)
11	N/A	N/A	N/A
12	Mean 2.2 (1.2), CI (1.9, 2.4) Median 1.8 (0.4, 5.4)	N/A	N/A
All	**Mean 1.7 (1.2), CI (1.6,1.7)** **Median 1.4 (<0.1-11)**	**Mean 1.8 (1.2), CI (1.7,1.9)** **Median 1.5 (<0.1-9.1)**	**Mean 1.5 (66), CI (1.4,1.5)** **Median 1.1 (<0.1-11)**

SD = standard deviation, CI = confidence interval, min = minimum, max = maximum, Obs. = observer, # = number, PAM = primary acquired melanosis, C-MIN = conjunctival melanocytic intraepithelial neoplasia, WHO = World Health Organization

SHADED RECTANGLES = **observers who use classification in routine pathology practice**

Table 4

Table 4. PAM, C-MIN, and WHO classification systems: Agreement between all observers

Classification system	Percent agreement (%)	Fleiss Kappa	95% CI	*P*-value
PAM (0 vs. 1 vs. 2)	55	0.37	0.35-0.39	<.0001
PAM (0 vs. 1-2)	69	0.37	0.34-0.40	<.0001
PAM (0-1 vs. 2)	76	0.58	0.55-0.61	<.0001
C-MIN (0-4 vs. 5-10)	67	0.52	0.49-0.56	<.0001
C-MIN (0-5 vs. 6-10)	67	0.52	0.48-0.55	<.0001
C-MIN (Radial spread)	38	0.11	0.08-0.14	<.0001
C-MIN (Vertical spread)	39	0.12	0.09-0.15	<.0001
C-MIN (Melanocyte atypia)	38	0.13	0.10-0.16	<.0001
WHO (0 vs. 1 vs. 2 vs. 3)	46	0.26	0.24-0.28	<.0001
WHO (0-1 vs. 2-3)	81	0.36	0.34-0.38	<.0001
WHO (0-2 vs. 3)	85	0.36	0.35-0.36	<.0001

PAM = primary acquired melanosis, C-MIN = conjunctival melanocytic intraepithelial neoplasia, WHO = World Health Organization, CI = confidence interval

PAM classification key [3]
0 = increased pigment in epithelial cells without melanocytic hyperplasia or atypia, melanocytic hyperplasia without cytomorphologic atypia
1 = PAM with cytomorphologic atypia (enlarged dendritic or polyhedral melanocytes), basilar hyperplasia
2 = PAM with cytomorphologic atypia, other (non-basilar hyperplasia, epithelioid morphology)

C-MIN scoring system is scored out of 10 [6]
Growth pattern / Radial (horizontal) spread
Nil (score 0), basal (score 1), pagetoid (score 2), isolated nests (score 3), confluent nests (score 4)
Vertical spread (suprabasilar extension)
Nil (score 0), 1-50% (score 1), 50-90% (score 2), >90% (score 3)
Melanocytic atypia (cumulative score 0-3)
Nucleus/cytoplasm <basal squamous cell (score 0), nucleus $\geq$ basal squamous cell (score 1), cytoplasm > basal squamous cell (score 1), Nucleoli and/or mitoses (score 1)

WHO classification key[1]
0 = increased pigment in epithelial cells without melanocytic hyperplasia or atypia/ conjunctival epithelial hypermelanosis (corresponds to PAM without atypia, C-MIN score = 0)
1 = low-grade conjunctival melanocytic intraepithelial lesion; predominantly basilar melanocytic hyperplasia without atypia or with mild atypia (dendritic or small polyhedral, non-epithelioid melanocytes) (corresponds to PAM without atypia or PAM with mild atypia, C-MIN score = 1- 2)
2 = high-grade conjunctival melanocytic intraepithelial lesion; significant non-basilar melanocytic hyperplasia involving <50% of epithelium without nests and/or melanocytes with epithelioid morphology, C-MIN score 3-5)
3 = melanoma in situ; atypical melanocytes with nesting and/or >50% full-thickness intraepithelial migration (corresponds to C-MIN score >5)

Percent agreement (%): number of agreement scores / total scores x100

Fleiss Kappa values interpretation:[12] < 0 Poor agreement, 0.01 – 0.20 Slight agreement, 0.21 – 0.40 Fair agreement, 0.41 – 0.60 Moderate agreement, 0.61 – 0.80 Substantial agreement, 0.81 – 1.00 Almost perfect agreement.

Table 5

Table 5. PAM, C-MIN, and WHO classification systems: Sensitivity, specificity, and diagnostic accuracy

Threshold	Sensitivity (%)	SD	Specificity (%)	SD	Distance to 0,1	SD	AUC (%)	SD (%)
PAM classification system								
0-1 vs 2	86	10	75	11	0.29	0.11	81	5
0 vs 1-2	95	4	38	18	0.62	0.17		
≥0	100	0	0	0	1.00	0.00		
C-MIN classification system								
0-9 vs 10	18	18	97	3	0.84	0.18	83	4
0-8 vs 9,10	32	21	93	4	0.69	0.19		
0-7 vs 8-10	48	25	89	4	0.54	0.23		
0-6 vs 7-10	58	26	86	7	0.47	0.22		
0-5 vs 6-10	72	20	81	12	0.37	0.16		
0-4 vs 5-10	83	11	73	16	0.35	0.13		
0-3 vs 4-10	89	7	63	23	0.40	0.21		
0-2 vs 3-10	92	3	53	24	0.48	0.24		
0-1 vs 2-10	96	4	38	25	0.63	0.24		
0 vs 1-10	96.	4	21	19	0.79	0.19		
≥0	100	0	0	0	1.00	0.00		
WHO classification system								
0-2 vs 3	41	27	92	6	0.60	0.26	83	6
0-1 vs 2-3	81	18	76	9	0.35	0.11		
0 vs 1-3	97	4	30	19	0.70	0.19		
≥0	100	0	0	0	1.00	0.00		

PAM = primary acquired melanosis, C-MIN = conjunctival melanocytic intraepithelial neoplasia, WHO = World Health Organization, SD = standard deviation; Distance to 0,1= threshold with optimal sensitivity and specificity, AUC = area under receiver operating characteristic curve

PAM classification key[3]

0 = increased pigment in epithelial cells without melanocytic hyperplasia or atypia, melanocytic hyperplasia without cytomorphologic atypia

1 = PAM with cytomorphologic atypia (enlarged dendritic or polyhedral melanocytes), basilar hyperplasia

2 = PAM with cytomorphologic atypia, other (non-basilar hyperplasia, epithelioid morphology)

C-MIN scoring system is scored out of 10[5]

Growth pattern / Radial (horizontal) spread

Nil (score 0), basal (score 1), pagetoid (score 2), isolated nests (score 3), confluent nests (score 4)

Vertical spread (suprabasilar extension)

Nil (score 0), 1-50% (score 1), 50-90% (score 2), >90% (score 3)

Melanocytic atypia (cumulative score 0-3)

Nucleus/cytoplasm <basal squamous cell (score 0), nucleus ≥ basal squamous cell (score 1), cytoplasm > basal squamous cell (score 1), Nucleoli and/or mitoses (score 1)

WHO classification key[1]

0 = increased pigment in epithelial cells without melanocytic hyperplasia or atypia/ conjunctival epithelial hypermelanosis (corresponds to PAM without atypia, C-MIN score = 0)

1 = low-grade conjunctival melanocytic intraepithelial lesion; predominantly basilar melanocytic hyperplasia without atypia or with mild atypia (dendritic or small polyhedral, non-epithelioid melanocytes) (corresponds to PAM without atypia or PAM with mild atypia, C-MIN score = 1- 2)

2 = high-grade conjunctival melanocytic intraepithelial lesion; significant non-basilar melanocytic hyperplasia involving <50% of epithelium without nests and/or melanocytes with epithelioid morphology, C-MIN score 3-5)

3 = melanoma in situ; atypical melanocytes with nesting and/or >50% full-thickness intraepithelial migration (corresponds to C-MIN score >5)

Table 6. PAM, C-MIN, and WHO classification: Scoring of conjunctival melanocytic intraepithelial lesions that recurred

Pt. #	PAM				C-MIN					WHO		
	BS	11 observers			BS	11 observers				12 observers		
		PAM 0 N (%)	PAM 1 N (%)	PAM 2 N (%)		C-MIN 0 N (%)	C-MIN 1-2 N (%)	C-MIN 3-4 N (%)	C-MIN 5-10 N (%)	WHO 0 N (%)	WHO 1 N (%)	WHO 2-3 N (%)
1	2	0	0	11 (100)	9	0	0	0	11 (100)	0	0	12 (100)
2	2	0	3 (27)	8 (73)	7	0	0	4 (36)	7 (64)	0	3 (25)	9 (75)
3	2	0	0	11 (100)	8	0	0	0	11 (100)	0	0	12 (100)
4	2	0	0	11 (100)	7	0	0	0	11 (100)	0	1 (8)	11 (92)
5	2	0	0	11 (100)	4	0	0	3 (27)	8 (73)		3 (25)	9 (75)
6	2	0	1 (9)	10 (91)	9	0	1 (9)	0	10 (91)	0	1 (8)	11 (92)
7	2	0	0	11 (100)	7	0	0	0	11 (100)	0	1 (8)	11 (92)
8	2	0	2 (18)	9 (82)	6	0	1 (9)	2 (18)	8 (73)	0	3 (25)	9 (75)
9	2	0	0	11 (100)	10	0	0	0	11 (100)	0	0	12 (100)
10	2	0	2 (18)	9 (82)	10	0	0	0	9 (100)*	0	0	12 (100)
11	2	0	0	11 (100)	10	0	0	0	11 (100)	0	0	12 (100)
12	2	0	0	11 (100)	9	0	0	0	11 (100)	0	0	12 (100)
13	1	8 (73)	2 (18)	1 (9)	0	4 (36)	5 (46)	2 (18)	0	4 (33)	8 (67)	0

Pt. = patient, # = number, BS = benchmark standard (based on their contribution to PAM and C-MIN classification systems and their experience with these systems, Dr. Robert Folberg's and Dr. Sarah Coupland's scores were used as benchmark standards for PAM and C-MIN scoring, respectively), PAM = primary acquired melanosis, C-MIN = conjunctival melanocytic intraepithelial neoplasia, WHO = World Health Organization, N (%) = number and percentage of observers that assigned a specific score, *2 observers did not score

PAM classification key[3]

0 = increased pigment in epithelial cells without melanocytic hyperplasia or atypia, melanocytic hyperplasia without cytomorphologic atypia
1 = PAM with cytomorphologic atypia (enlarged dendritic or polyhedral melanocytes), basilar hyperplasia
2 = PAM with cytomorphologic atypia, other (non-basilar hyperplasia, epithelioid morphology)

C-MIN scoring system is scored out of 10[6]

Growth pattern / radial (horizontal) spread
Nil (score 0), basal (score 1), pagetoid (score 2), isolated nests (score 3), confluent nests (score 4)
Vertical spread (suprabasilar extension)
Nil (score 0), 1-50% (score 1), 50-90% (score 2), >90% (score 3)
Melanocytic atypia (cumulative score 0-3)
Nucleus/cytoplasm <basal squamous cell (score 0), nucleus $\geq$ basal squamous cell (score 1), cytoplasm > basal squamous cell (score 1), Nucleoli and/or mitoses (score 1)

WHO classification key[1]

0 = increased pigment in epithelial cells without melanocytic hyperplasia or atypia/ conjunctival epithelial hypermelanosis (corresponds to PAM without atypia, C-MIN score = 0)
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2 = high-grade conjunctival melanocytic intraepithelial lesion; significant non-basilar melanocytic hyperplasia involving <50% of epithelium without nests and/or melanocytes with epithelioid morphology, C-MIN score 3-5)
3 = melanoma in situ; atypical melanocytes with nesting and/or >50% full-thickness intraepithelial migration (corresponds to C-MIN score >5)

Table 7. Consensus proposed histologic criteria for integrated classification of conjunctival melanocytic intraepithelial lesions.

WHO grade	Definition	Histologic description
Low-grade	Benign melanosis	Conjunctival hypermelanosis (increased pigment in epithelial cells without melanocytic hyperplasia or atypia) Melanocytic hyperplasia without atypia (melanocytes with condensed round nuclei, smaller than basal epithelial cell, inconspicuous nucleoli, and inconspicuous cytoplasm)
	Low-grade CMIL	Predominantly basilar melanocytic hyperplasia with mild atypia (dendritic or small polyhedral, non-epithelioid melanocytes with irregular nuclear contours, inconspicuous nucleoli, and inconspicuous or scant cytoplasm)
High-grade	High-grade CMIL	Significant non-basilar hyperplasia of atypical melanocytes involving <50% of epithelium without nests and/or melanocytes with epithelioid morphology
	Melanoma in situ	Atypical melanocytes with nesting and/or >50% full-thickness intraepithelial migration

WHO = World Health Organization, CMIL = conjunctival melanocytic intraepithelial lesion

Figure 1

Click here to access/download;Figure - label number in Description field; no PDFs;Figure 1 CMIL clinical and histo

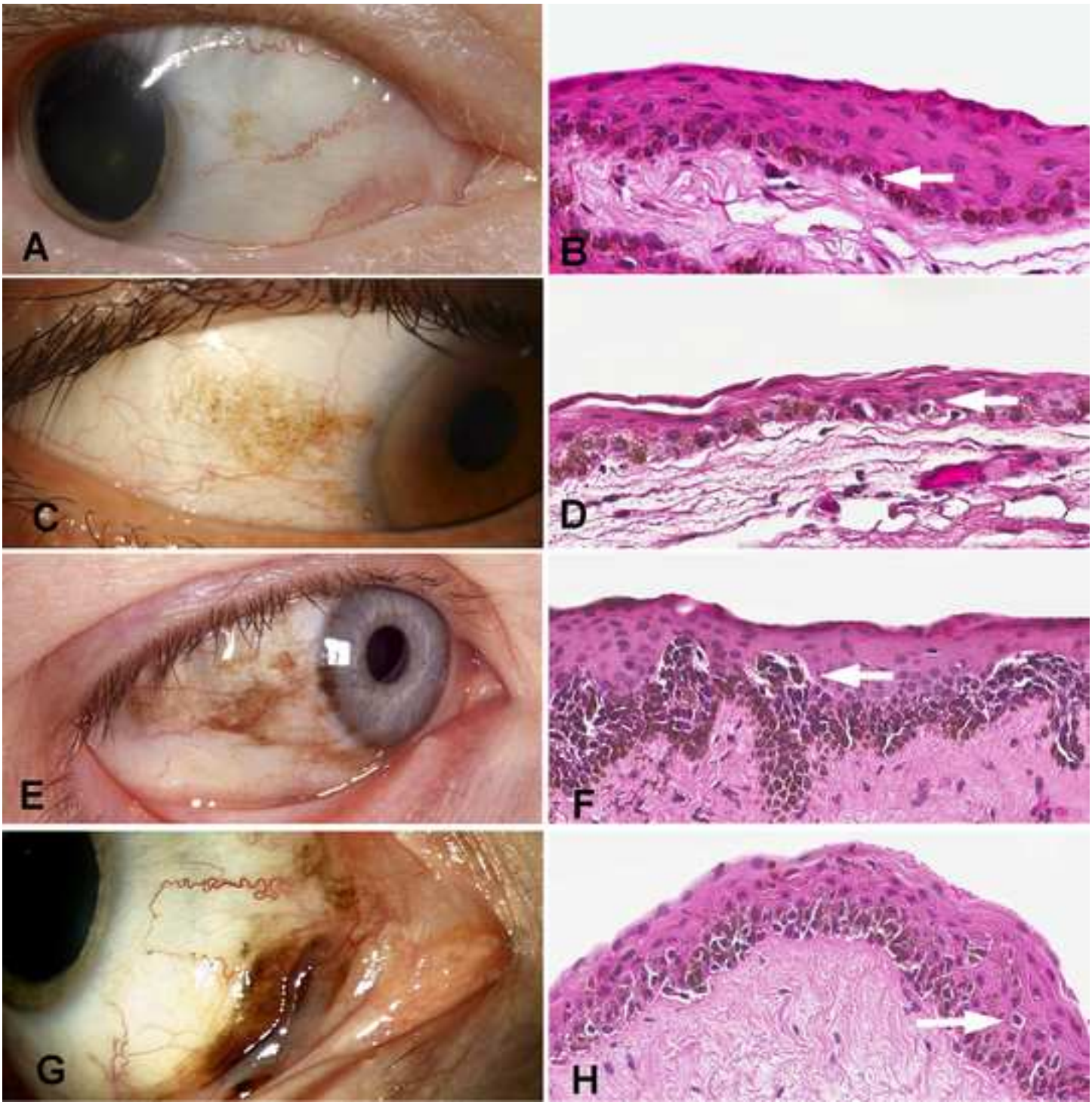

Figure 2

Click here to access/download;Figure - label number in Description field; no PDFs;Figure 2 CMIL challenging cases.jpg

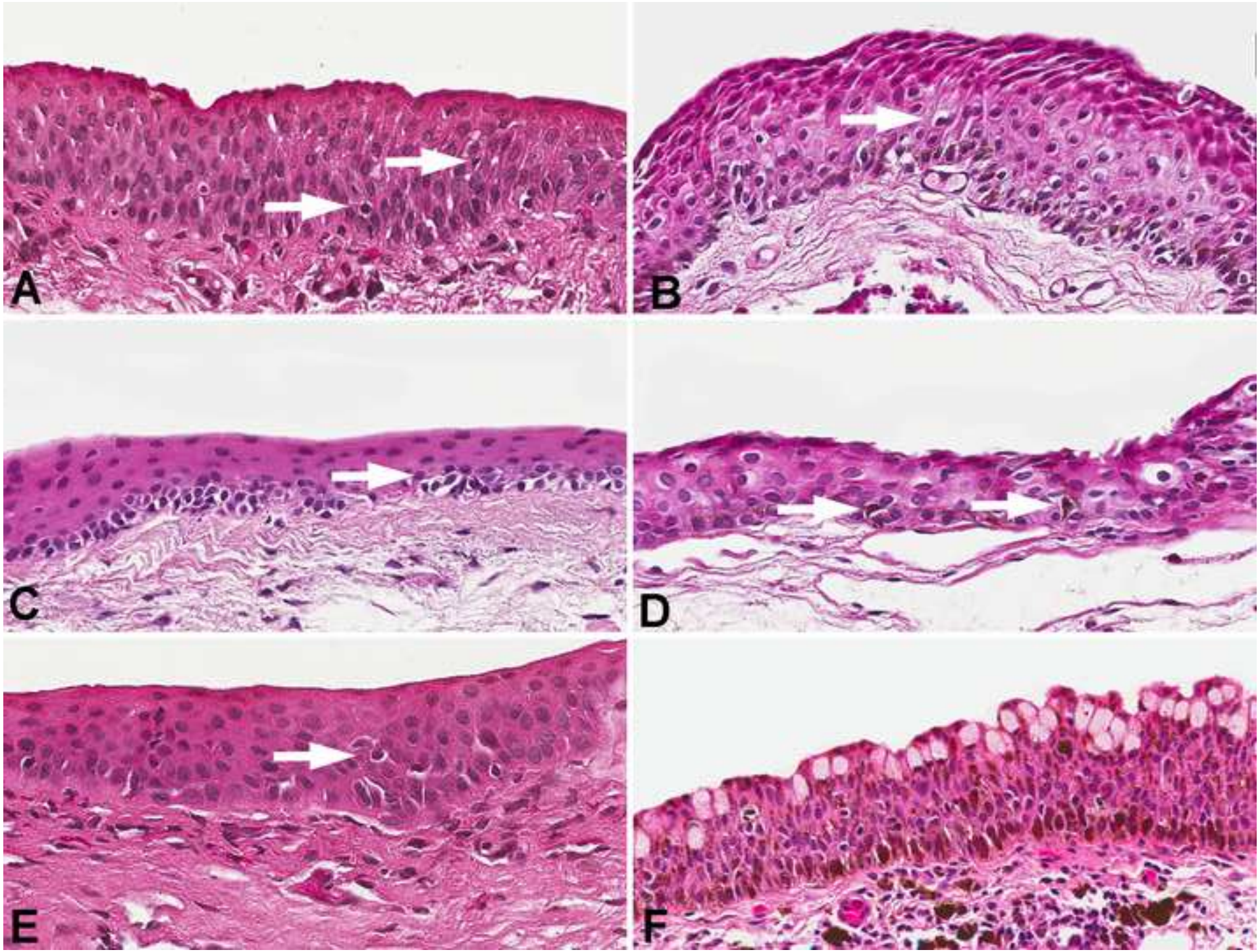

Figure 3

Click here to access/download;Figure - label number in Description field; no PDFs;Figure 3 CMIL ROCs.jpg

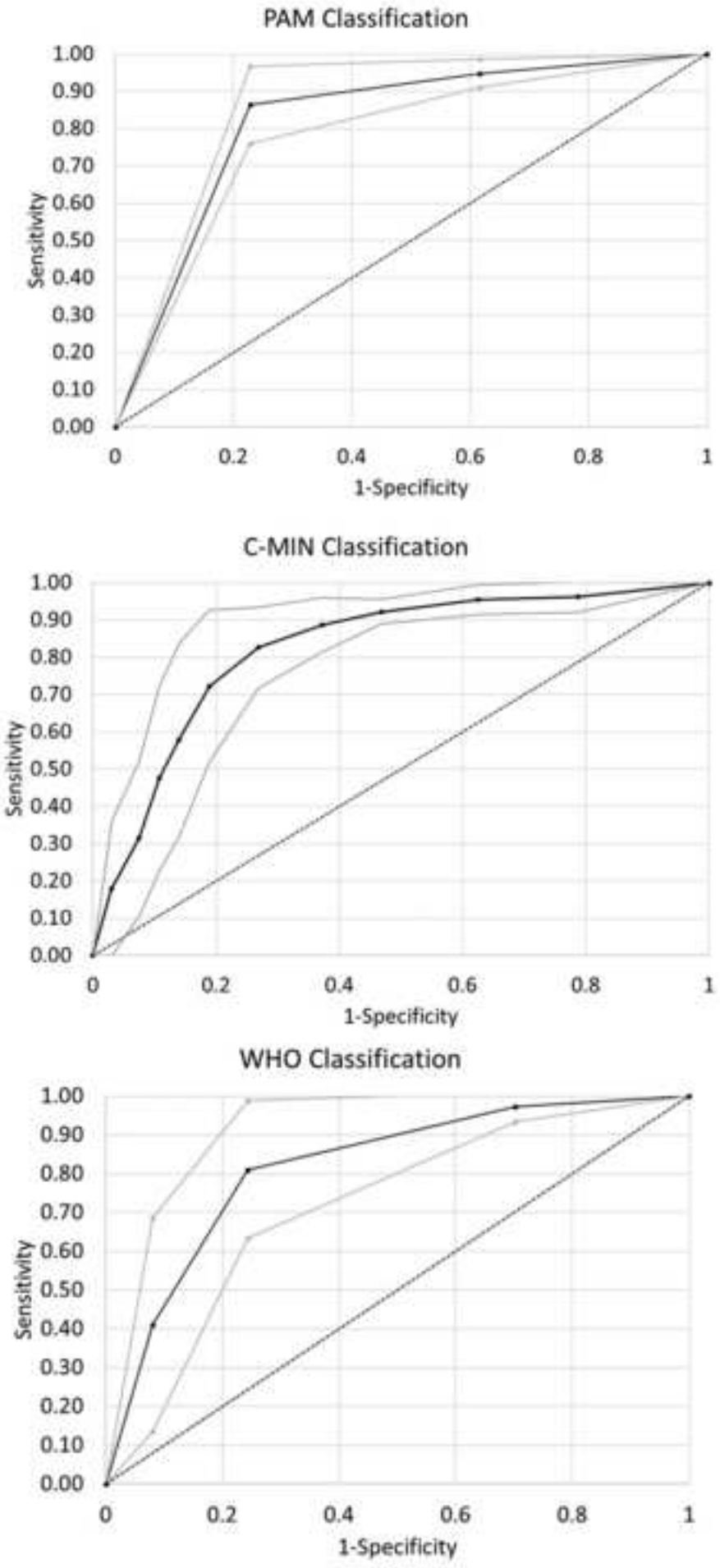

Table of Contents Statement

Manuscript: "*Validation of the newly proposed WHO classification system for conjunctival melanocytic intraepithelial lesions: a comparison with the 'C-MIN' and 'PAM' classification schemes*"

This study was designed to assess the inter-observer variability, sensitivity, specificity, and accuracy of the recently developed WHO classification system for conjunctival melanocytic intraepithelial lesions by comparing it to the two most commonly used classification systems, PAM and C-MIN. The three classification systems had comparable sensitivity, specificity, and accuracy of in their ability to identify lesions with potential for recurrence. WHO classification system is appropriate for pathologic evaluation of conjunctival melanocytic intraepithelial lesions.

VALIDATION OF THE NEWLY PROPOSED WHO CLASSIFICATION SYSTEM FOR CONJUNCTIVAL MELANOCYTIC INTRAEPITHELIAL LESIONS: A COMPARISON WITH THE 'CMIN' AND 'PAM' CLASSIFICATION SCHEMES

HIGHLIGHTS

- Validation of the WHO classification for conjunctival melanocytic intraepithelial lesions
- PAM, C-MIN, and WHO classification schemes have accuracy of 81-83%
- The inter-observer agreement is 81% for low-grade vs high-grade CMIL
- Low-grade lesions present greatest interpretative challenge with all three systems

Tatyana Milman, MD, Biosketch

Tatyana Milman, MD, obtained her medical degree from the University of Pennsylvania School of Medicine, followed by Ophthalmology Residency at Rutgers – New Jersey Medical School and an Ophthalmic Pathology fellowship at Wills Eye Hospital. Dr. Milman completed a second residency in Anatomic Pathology at the University of Pennsylvania. She currently is an Associate Professor of Ophthalmology and Pathology at Thomas Jefferson University and Wills Eye Hospital. Her research interests include clinical-pathologic correlations in ophthalmic pathology.

Maya Eiger-Moscovich, MD, Biosketch

Maya Eiger-Moscovich, MD, obtained her medical degree from Sackler Faculty of Medicine, Tel Aviv University, Israel, and completed her Ophthalmology Residency at Rabin Medical Center, Israel. She completed a fellowship in Ocular Oncology and Ophthalmic Pathology, both at the Wills Eye Hospital. Dr. Maya Eiger-Moscovich will pursue a carrier in Ocular Oncology and Pathology at Hadassah Medical Center in Jerusalem, Israel. Her research interests include ocular oncology and ophthalmic pathology.

Biosketch Photo: Milman

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Biosketch Photo: Eiger-Moscovich

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Supplemental Table 1. PAM classification: Agreement between each observer and the benchmark standard*

Obs. #	PAM (0, 1, 2) Cohen Kappa (CI)	PAM (0 vs. 1-2) Cohen Kappa (CI)	PAM (0-1 vs. 2) Cohen Kappa (CI)	*P*-value
1	0.41 (0.27-0.54)	0.28 (0.13-0.43)	0.66 (0.49-0.83)	
2	0.38 (0.24-0.53)	0.35 (0.16-0.53)	0.60 (0.41-0.78)	
3	0.22 (0.07-0.38)	0.22 (-0.03-0.46)	0.31(0.11-0.51)	
4	0.43 (0.27-0.58)	0.33 (0.12-0.54)	0.70 (0.53-0.86)	
5	0.33 (0.16-0.50)	0.33 (0.09-0.57)	0.43 (0.23-0.64)	
6	0.22 (0.05-0.39)	0.11 (-0.13-0.35)	0.36 (0.15-0.58)	
7	0.39 (0.23-0.54)	0.25 (0.05-0.46)	0.61 (0.43-0.79)	
8	0.47 (0.30-0.63)	0.42 (0.16-0.68)	0.50 (0.30-0.70)	
9	0.21 (0.06-0.35)	0.34 (0.17-0.51)	0.39 (0.20-0.58)	
10	0.30 (0.16-0.43)	0.29 (0.15-0.43)	0.51 (0.32-0.70)	
11	0.37 (0.23-0.51)	0.24 (0.08-0.40)	0.66 (0.49-0.83)	
All	**0.34 (0.28-0.40)**	**0.27 (0.23-0.32)**	**0.53 (0.44-0.61)**	**<.001**

*Dr. Robert Folberg's scores were used as "PAM benchmark standard" based on his contribution to the PAM classification system and his experience with this system.
PAM = primary acquired melanosis, Obs. = observer, # = number, CI = 95% confidence interval

All = mean Kappa (95% confidence interval)
P-value for the non-parametric Friedman test comparing the means of the 3 Kappa statistics

PAM classification key[3]
0 = increased pigment in epithelial cells without melanocytic hyperplasia or atypia, melanocytic hyperplasia without cytomorphologic atypia
1 = PAM with cytomorphologic atypia (enlarged dendritic or polyhedral melanocytes), basilar hyperplasia
2 = PAM with cytomorphologic atypia, other (non-basilar hyperplasia, epithelioid morphology)

Kappa values Interpretation:[12] < 0 Poor agreement, 0.01 – 0.20 Slight agreement, 0.21 – 0.40 Fair agreement, 0.41 – 0.60 Moderate agreement, 0.61 – 0.80 Substantial agreement, 0.81 – 1.00 Almost perfect agreement.

Supplemental Table 2. C-MIN classification: Agreement between each observer and the benchmark standard*

Obs. #	C-MIN (0-4 vs. 5-10) Cohen Kappa (CI)	C-MIN (0-5 vs. 6-10) Cohen Kappa (CI)	Radial spread Cohen Kappa (CI)	Vertical spread Cohen Kappa (CI)	Melanocyte atypia Cohen Kappa (CI)
1	0.79 (0.64-0.94)	0.81 (0.66-0.96)	0.49 (0.35-0.62)	0.47 (0.32-0.62)	0.47 (0.32-0.62)
2	0.63 (0.46-0.80)	0.61 (0.43-0.80)	0.26 (0.13-0.39)	0.50 (0.35-0.64)	0.50 (0.35-0.64)
3	0.59 (0.39-0.79)	0.24 (0.01-0.46)	0.22 (0.11-0.33)	0.20 (0.07-0.33)	0.20 (0.07-0.33)
4	0.80 (0.65-0.94)	0.81 (0.66-0.96)	0.35 (0.20-0.49)	0.53 (0.39-0.68)	0.53 (0.39-0.68)
5	0.37 (0.22-0.51)	0.45 (0.28-0.61)	0.21 (0.10-0.32)	0.14 (0.05-0.22)	0.14 (0.05-0.22)
6	0.62 (0.44-0.81)	0.67 (0.48-0.86)	0.28(0.11-0.46)	0.21 (0.03-0.38)	0.21 (0.03-0.38)
7	0.59 (0.41-0.77)	0.57 (0.36-0.79)	0.23 (0.09-0.36)	0.18 (0.04-0.32)	0.18 (0.04-0.32)
8	0.49 (0.33-0.64)	0.56 (0.39-0.73)	0.14 (0.02-0.25)	0.10 (0.04-0.16)	0.10 (0.04-0.16)
9	0.77 (0.62-0.93)	0.66 (0.47-0.86)	0.20 (0.07-0.33)	0.30 (0.15-0.45)	0.30 (0.15-0.45)
10	0.67 (0.48-0.86)	0.63 (0.43-0.83)	0.22 (0.10-0.35)	0.33 (0.23-0.42)	0.33 (0.23-0.42)
11	0.60 (0.41-0.80)	0.73 (0.55-0.90)	0.44 (0.30-0.58)	0.53 (0.39-0.67)	0.53 (0.39-0.67)
All	**0.64 (0.56-0.711)**	**0.61 (0.51-0.71)**	**0.26 (0.20-0.33)**	**0.31 (0.21-0.41)**	**0.26 (0.18-0.35)**
	P-value = 1.00		*P*-value = 0.25		

* Dr. Sarah Coupland's scores were used as "C-MIN benchmark standard" based on her contribution to the C-MIN classification system and her experience with this system.
Rows highlighted in gray denote observers who use C-MIN classification in routine practice.
C-MIN = conjunctival melanocytic intraepithelial neoplasia, Obs. = observer, # = number, CI = 95% confidence interval
All = mean Kappa (95% confidence interval)

C-MIN scoring system is scored out of 10[6]
Growth pattern / Radial (horizontal) spread
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Vertical spread (suprabasilar extension)
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Melanocytic atypia (cumulative score 0-3)
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Kappa values Interpretation:[12] < 0 Poor agreement, 0.01 – 0.20 Slight agreement, 0.21 – 0.40 Fair agreement, 0.41 – 0.60 Moderate agreement, 0.61 – 0.80 Substantial agreement, 0.81 – 1.00 Almost perfect agreement.

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Credit Author Statement:

Study design: TM, RCE, SEC, RF, HSM, HEG, CE, SH, CAH, MCHC, KL
Data collection: MEM, TM, RCE, CLS
Data analysis and interpretation: TM, MEM, RKH, RCE, SEC, RF, HSM, HEG, CE, SH, CAH, MCHC, KL, SC, QZ, JS, TRS
Manuscript drafting and final review: TM, MEM, RKH, RCE, SEC, RF, HSM, HEG, CE, SH, CAH, MCHC, KL, SC, QZ, JS, TRS
Grant and laboratory support: TM, RCE
Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved: TM, RCE, CLS, MEM