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Ki67 proliferation index in medullary thyroid carcinoma: a comparative study of multiple counting methods and validation of image analysis and deep learning platforms

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Database management and statistics: BX

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Abstract (250 and structured for histopathology)

Background: The International Medullary Thyroid Carcinoma Grading System (IMTCGS) introduced in 2022 mandates evaluation of Ki67 proliferation index to assign a histologic grade for medullary thyroid carcinoma. However, manual counting remains a tedious and time-consuming task.

Methods: We aimed to evaluate the performance of three other counting techniques for Ki67 index, eyeballing by a trained experienced investigator, a machine learning-based deep learning algorithms (DeepLIIF), and an image analysis software with internal thresholding (Leica Aperio Nuclear) compared to the gold standard manual counting in a large cohort of 260 primarily resected medullary thyroid carcinoma.

Results: Ki67 proliferation index generated by all three methods correlate near-perfectly with the manual Ki67 index with kappa values ranging from 0.884 to 0.979 and inter-class correlation coefficients ranging from 0.969 to 0.983. Discrepant Ki67 results were only observed in cases with borderline manual Ki67 readings ranging from 3% to 7%. Medullary thyroid carcinomas with a high Ki67 index ($\geq 5\%$) determined using any of the four methods were associated with significantly decreased disease-specific survival and distant metastasis-free survival.

Conclusions: We herein validate a machine learning-based deep-learning platform and an image analysis software with internal thresholding to generate accurate automatic Ki67 proliferation indexes in medullary thyroid carcinoma. Manual Ki67 count remains useful when facing a tumor with a borderline Ki67 proliferation index of 3%-7%.

Introduction

Medullary thyroid carcinoma is a neuroendocrine carcinoma derived from parafollicular C-cells ¹. It accounts for approximately 2% of all thyroid malignancies and 8% of thyroid cancer-related mortality ¹. In 2022, we established an international MTC consortium, developed the International Medullary Thyroid Carcinoma Grading System (IMTCGS), and validated its prognostic values in multivariate survival analysis ². This grading system incorporates three pathologic parameters, namely mitotic index, Ki67 proliferation index, and tumor necrosis ². Subsequent studies have independently validated the prognostic values of IMTCGS ³. Therefore, Ki67 proliferation index has become an essential pathologic parameter to report for accurate medullary thyroid carcinoma grading and risk stratification.

Williams et al. recently demonstrated excellent intraobserver agreement of manual assessment of Ki67 proliferation index with a kappa value of 0.86 in a cohort of 44 medullary thyroid carcinoma ⁴. However, manual Ki67 count is known to be tedious and time consuming. Various digital image analysis platforms and machine learning (ML)-based algorithms have shown to be highly sensitive and specific to obtain an automatic reading of Ki67 index in pancreatic neuroendocrine neoplasms and breast carcinomas ⁵⁻¹¹. The utility of these platforms in determining Ki67 index in medullary thyroid carcinoma is yet to be determined.

In this large-scale multicenter retrospective study of 260 patients with primarily resected medullary thyroid carcinoma, we compared the performance of four different evaluation methods aiming to validate an image analysis and machine learning -based deep learning platforms for automatic determination of Ki67 proliferation index in medullary thyroid carcinoma.

Materials and methods

Study cohort

This retrospective cohort study included 260 patients with primary MTC who underwent surgical resection at 5 tertiary centers (University of Bologna Medical Center [UB], Bologna, Italy: n=32; Memorial Sloan Kettering Cancer Center [MSKCC], New York, NY, USA: n=54; Institut Gustave Roussy, Villejuif, France: n=70; Royal North Shore Hospital, Sydney, Australia: n=63, and Emory University Hospital Midtown [EU], Atlanta, GA, USA: n=41). All cases were included in previously published studies from our group ^{2, 12}.

Assessment of Ki67 proliferation index

Ki67 proliferation index was determined by a single author (BX) using four methods: manual counting, one image analysis system, one deep learning platform, and eyeballing estimates. The results of manual counting were considered as the gold standard. The eyeballing, image analysis, and deep learning digital assessment was performed blinded to the gold standard manual readings. A JPG image was obtained at the hotspot of each tumor at 40X magnification using whole slide images (n=174) or glass slides (n=86). The Ki67 proliferation index was obtained using the same JPG image per tumor for all four methods. A training set of 20 Ki67 images with known number of positive and negative cells was reviewed prior to the actual eyeballing assessment.

The manual count of positive and negative cells was determined using a manual annotation tool in the QuPath platform (version 0.3.2, QuPath developers, The University of Edinburgh, Edinburgh, United Kingdom) ¹³.

Two image analysis and ML-based quantification platforms were used to obtain automatic Ki67 reading. The first was DeepLIIF (deepliif.org), MSKCC, New York, NY, USA), a free, online virtual multiplex immunofluorescence restaining cloud-native platform for immunohistochemistry images. This experimental platform was previously validated for nuclear and non-nuclear immunohistochemical markers ^{14, 15}. The second method was based on Leica Aperio Nuclear image analysis software (Leica Biosystems Imaging Inc, Vista, CA, USA) that was internally thresholded for nuclear immunohistochemical marker expression and was retrospectively validated for Ki67 proliferation index in breast cancer ¹⁶.

The study was performed with the default parameters set in both Leica Aperio Nuclear image analysis and DeepLIIF AI platforms.

The actual Ki67 proliferation index (%) was recorded for each method, and subsequently further classified into two categories: high Ki67 ($\geq 5\%$) and low Ki67 ($< 5\%$) using the 5% threshold established in the IMTCGS study ².

Statistical analyses

All statistical analyses were performed using SPSS software, version 24.0 (IBM, Armonk, NY, USA). The concordance between each method and the gold standard was determined using Fleiss's kappa statistics. A kappa value of 0.01–0.20 indicated slight agreement, 0.21–0.40 fair agreement, 0.41–0.60 moderate agreement, 0.61–0.80 substantial agreement

and 0.81–0.99 near-perfect agreement. The Ki67 proliferation indices from each respective method were compared using interclass correlation coefficient (ICC) with the gold standard manual Ki67 quantification for each tumor.

The prognostic values of high Ki67 and low Ki67 were determined using log rank test for disease-specific survival and distant metastasis-free survival. The frequency of *RET* germline mutations, *RET* somatic mutations, *RET* p.M918T somatic mutations, and *RAS* somatic mutations was calculated and compared according to Ki67 high and low status.

Results

Agreement of various platforms to assess Ki67 proliferation index in medullary thyroid carcinoma

The median and mean numbers of cells counted per tumor were 1126 and 1178 cells respectively (range: 331 to 2970). The frequency of high ($\geq 5\%$) Ki67 proliferation index was 10.4% (n=27), 11.2% (n=29), 10.0% (n=26), and 11.9% (n=31) respectively using manual, eyeballing, DeepLIF, and module #2. All three methods showed near-perfect agreement compared with the gold standard manual Ki67 counts, and the automatic reading obtained using DeepLIF platform achieved the highest kappa value (eyeballing: kappa = 0.960, $p < 0.001$; DeepLIF: kappa = 0.979, $p < 0.001$; Leica Aperio: kappa = 0.884, $p < 0.001$, **Table 1** and **Figure 1A**).

Cases with discrepant Ki67 results were only observed in tumors with a borderline manual Ki67 proliferation index between 3% and 7%. These borderline cases were relatively infrequent, accounting for 8.5% of all medullary thyroid carcinomas included in the study. When limiting Fleiss's kappa analysis to this subgroup only, the kappa values decreased to 0.804 (near perfect agreement), 0.891 (near perfect agreement), and 0.436 (moderate agreement) respectively for eyeballing, DeepLIF, and Leica Aperio comparing with the gold standard manual Ki67.

There was also excellent reliability of the actual Ki67 proliferation index obtained using eyeballing or deep learning platforms when compared with the gold standard manual Ki67 index. The inter-class correlation coefficients were 0.983 (95% confidence interval 0.979 – 0.987, $p < 0.001$) for eyeballing, 0.978 (95% confidence interval 0.972 – 0.983, $p < 0.001$) for DeepLIF, and 0.968 (95% confidence interval 0.857 – 0.975, $p < 0.001$) for Leica Aperio (**Table 1** and **Figure 1B**).

Correlation of Ki67 proliferation index with outcomes and *RET*/*RAS* mutation status

In the study cohort, 252 patients had follow-up available with a median follow up time of 43 months (range: 0.4 – 232 months). The high Ki67 subgroup consistently associated with decreased disease-specific survival and distant metastasis-free survival regardless of the counting methods used (log rank test, $p < 0.001$ for all comparisons, **Figure 1G**).

The *RET* germline mutation, *RET* somatic mutation, and *RAS* mutations status were known in 252, 208, and 206 patients respectively. There was no significant correlation between Ki67 proliferation index and underlying *RET*/*RAS* mutations, including *RET* germline mutations, *RET* somatic mutations, *RET* p.M918T somatic mutations, and *RAS* somatic mutations (Fisher's exact test, $p > 0.05$, data not shown).

Discussions

Recently, the International Medullary Thyroid Carcinoma Grading System, a two-tiered grading system including a Ki67 cutoff of 5%, was proposed and validated for medullary thyroid carcinoma^{2,3}, rendering determination of Ki67 proliferation index an essential step in the pathology evaluation of this tumor type. In the current study, we demonstrated that ML-based deep learning and imaging analysis platforms as well as eyeballing evaluation by a trained experienced evaluator can generate highly accurate and reliable Ki67 proliferative index readings necessary for grading and for risk stratification of medullary thyroid carcinoma.

The perceived advantages and disadvantages of each counting method are summarized in **Table 2**. Manual counting to date is considered as the gold standard method to count Ki67 proliferation index in neuroendocrine neoplasms^{5,10,11}. It is highly accurate, readily available, permitting inclusion/exclusion of specific areas, and allowing case-based adjustment of positive threshold. The printed counting sheets and/or the snapshots of manual counting via imaging software (e.g. QuPath) can be stored for record purposes. The key disadvantage of manual counting is the time consumption. For the current study, an average of 80 cells could be counted per minute and assessing each case required 15 to 20 minutes on average.

Eyeballing is the fastest method among all platforms evaluated. It does not require a digital image (snapshot or whole slide image) and can be readily performed on glass slides using microscope. The caveat is that this method is heavily

operator dependent. A training set of representative images with known positive and negative cell counts may be useful in improving the performance of eyeballing.

Several recent studies demonstrated that deep learning algorithms could be used to generate automatic Ki67 proliferation index with high sensitivity and specificity in breast carcinomas and pancreatic neuroendocrine neoplasms ⁶⁻⁹. Both breast carcinomas and pancreatic neuroendocrine neoplasms use three-tiered Ki67 cutoffs that are different from the two-tiered 5% cutoff of medullary thyroid carcinoma. Recently, we have validated an ML-based deep learning platform DeepLIIF freely available online deepliif.org^{14, 15} and a commercially available image analysis software (Leica Aperio image analysis) internally validated for automatic Ki67 reading in breast carcinoma. We herein validated both methods in assessing Ki67 proliferation index in medullary thyroid carcinoma. Both platforms generated highly concordant results compared with manual counting with near perfect kappa values and inter-class correlation coefficients.

The two platforms have certain differences. The DeepLIIF online platform is freely available to public. Data analyzed are stored on the DeepLIIF server and can be re-accessed through a case-specific web link. The results and images are also downloadable for storage. Various parameters, including segmentation, size of cells, and thresholds of positivity, are easily adjustable through sliding bars and the adjusted results are available in real time. It currently only allows snapshots in more than 150 input image formats. DeepLIIF whole slide image analysis tool and viewer are under development.

The Leica Aperio Nuclear image analysis software is applicable for both whole slide images and static images (e.g. snapshots). Among the four methods included in this study, it is the only system that provides percentage of positivity for each intensity, being 1+ (weak), 2+ (moderate), and 3+ (strong). In the context of evaluating Ki67 proliferation index, such feature is useful to exclude nonspecific weak (1+) nuclear or background brown signals as nonspecific staining rather than true positive staining. One drawback is that the software is only commercially available and must be internally thresholded to ensure appropriate performance at individual laboratories. It also has poor segmentation and can easily merge neighboring cells (**Figure 1F**). Additionally, although the parameters of this image analysis system are adjustable, it is less accessible compared to the DeepLIIF interface.

One caveat of using eyeballing or deep learning algorithms to determine Ki67 in medullary thyroid carcinoma is the relative inaccuracy in cases with borderline Ki67 indexes. In the current study, discrepancies were noted in cases with a Ki67 proliferation index of 5%±2% (i.e. 3% to 7%). In such cases, we recommend using manual counting to accurately determine the Ki67 proliferation index and to subsequently grade the tumor, similar to the approach recommended by the College of American Pathologists (CAP) for borderline cases of pancreatic neuroendocrine neoplasms ¹⁷. Fortunately, the frequency of these borderline cases is relatively low in medullary thyroid carcinoma, being 8.5% in the current study. The discrepant cases could also be corrected via the size gating slider in DeepLIIF, but our study was performed with default parameters using both Leica Aperio Nuclear image analysis and DeepLIIF AI platforms.

Ki67 proliferation index was shown to be a prognostic factor to predict overall survival, disease-specific survival, distant metastasis-free survival, and locoregional recurrence-free survival in medullary thyroid carcinoma ^{2, 18, 19}. Similarly, we confirmed that high (≥5%) Ki67 was associated with decreased disease-specific survival and distant metastasis-free survival, regardless of the scoring methods used.

In conclusions, we herein demonstrate that ML-based deep learning algorithms, image analysis software, and eyeballing evaluation by a trained experienced evaluator can accurately assess Ki67 proliferation index in medullary thyroid carcinoma comparable to the time-consuming manual counting. In the small percentage of borderline cases with a Ki67 proliferation index of 3% to 7%, manual Ki67 counts remains the most reliable method to grade and accurately stratify risk in medullary thyroid carcinoma.

Table 1. Agreement of Ki67 proliferation index in medullary thyroid carcinoma. Manual Ki67 counting was the gold standard reference. The raw Ki67 proliferation index and its categorical subgroups (high Ki67 $\geq 5\%$ vs. low Ki67 $< 5\%$) for each tumor were determined using three additional evaluation methods: eyeballing, DeepLIIF - a deep learning platform, and Leica Aperio Nuclear image analysis software.

ICC: inter-class correlation coefficient.

Ki67 platforms	High ($\geq 5\%$) Ki67, n (%)	Kappa	Kappa in subgroup with 3%-7% Ki67	ICC
Manual counting	27 (10.4%)	Reference (gold standard)		
Eyeballing	29 (11.2%)	0.960	0.804	0.983
DeepLIIF	26 (10.0%)	0.979	0.891	0.978
Leica Aperio	31 (11.9%)	0.884	0.436	0.968

Table 2. Comparison of advantages and disadvantages of each evaluation method for Ki67 in medullary thyroid carcinoma.

	Manual	Eyeballing	DeepLIIF	Leica Aperio
Estimate time per case	Time-consuming: 15 to 20 minutes	Short: < 1 minute		
Accuracy	Highly accurate (gold standard)	Operator dependent (training may improve the performance)	Highly accurate: showed highest concordance	Accurate: discrepancy is only noted in borderline cases with Ki67 index of 5%±2%
Availability	Widely available		Free online platform https://deepliif.org/	Commercial, requires internal thresholding
Including/excluding specific area(s)	Permitted			
Format requirements for the Images	Static images (e.g. snapshots) or whole slide images Inaccurate on glass slides	Variable formats: H&E slide, snapshots, whole slide images	Static images (whole slide image analysis: not yet available)	Snapshots or whole slide images
Adjustable thresholds for positivity	Adjustable but subjective		Easily adjustable: sliding scale to adjust the thresholds for segmentation, size, and positivity	Less adjustable: thresholds have been set through machine learning, but can be manually adjusted
Intensity assessment	No		Yes: virtual multiplex retained results	Yes: 1+, 2+, and 3+
Data and results storage	Can be stored on local data storage devices or as hard copies	Not available	Stored on DeepLIIF cloud server with a unique web link per case. Downloadable to local data storage devices	Can be stored on local data storage devices

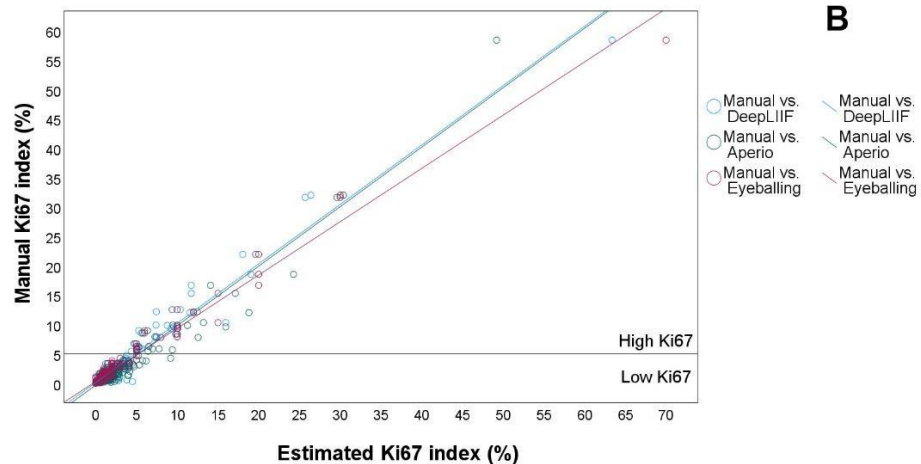
Figure legends

Figure 1. Evaluation of Ki67 proliferation index in medullary carcinoma using various platforms. (A) Heatmap plotting demonstrating classification of high ($\geq 5\%$) and low ($< 5\%$) Ki67 indexes using manual counting, eyeballing, and two image analysis and ML-based quantification platforms (DeepLIF and Leica Aperio Nuclear image analysis software). All discrepant readings occur in cases with a manual Ki67 count of 3% to 7%. (B) Scattered plot illustrating correlation of raw Ki67 index (%) between manual method (Y-axis) and the other 3 methods (X-axis, DeepLIF: green, Leica Aperio: blue, Eyeballing: red). (C-F) A medullary thyroid carcinoma with high Ki67 proliferation index. (C) Snapshot of the Ki67 proliferation index at the hotspot. (D) Manual counting: red circles are positive cells, yellow circles are negative cells. (E) DeepLIF overlaid image: red-contoured cells are positive, blue-contoured cells are negative. (F) Leica Aperio overlaid image: negative cells are blue, positive cells are yellow (intensity: 1+), orange (intensity: 2+) and brown (intensity: 3+). (G) Kaplan Meier curves for disease specific survival (DSS, top row) and distant metastasis free survival (DMFS, bottom row, log rank test, $p < 0.001$ for all comparisons). Medullary thyroid carcinomas are categorized as high ($\geq 5\%$) Ki67 and low ($< 5\%$) ki67 subgroups using the four different counting methods.

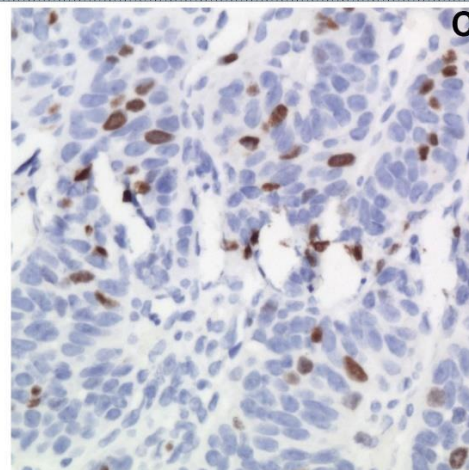
Ki67 proliferation index $\geq 5\%$ $< 5\%$

A

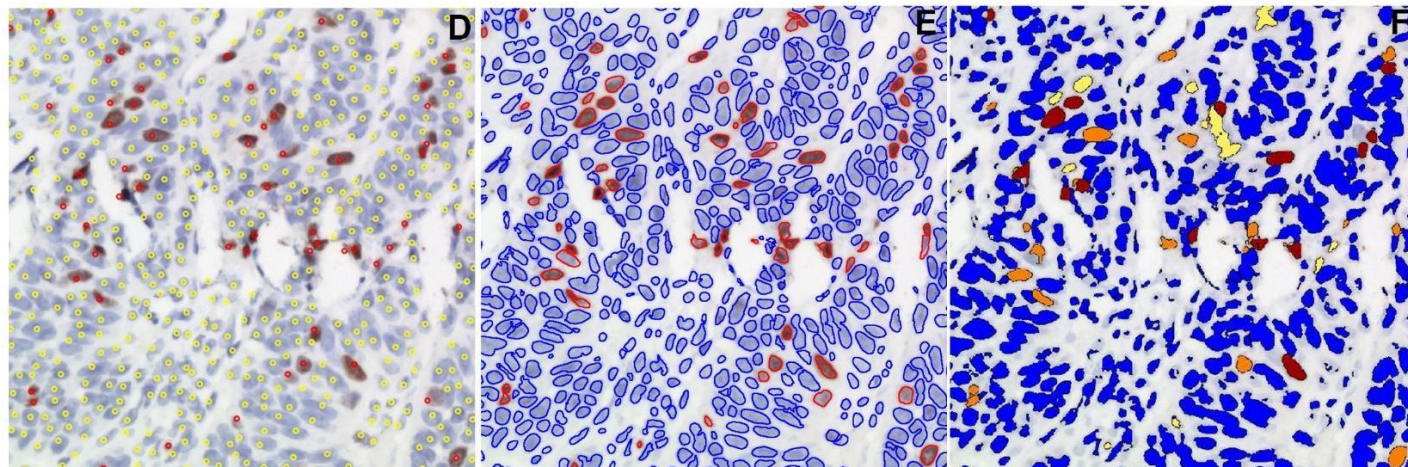
	>7%	3-7%	<3%
Manual	15	10	75
Eyeballing	15	10	75
DeepLIIF	15	10	75
Aperio	15	10	75



B



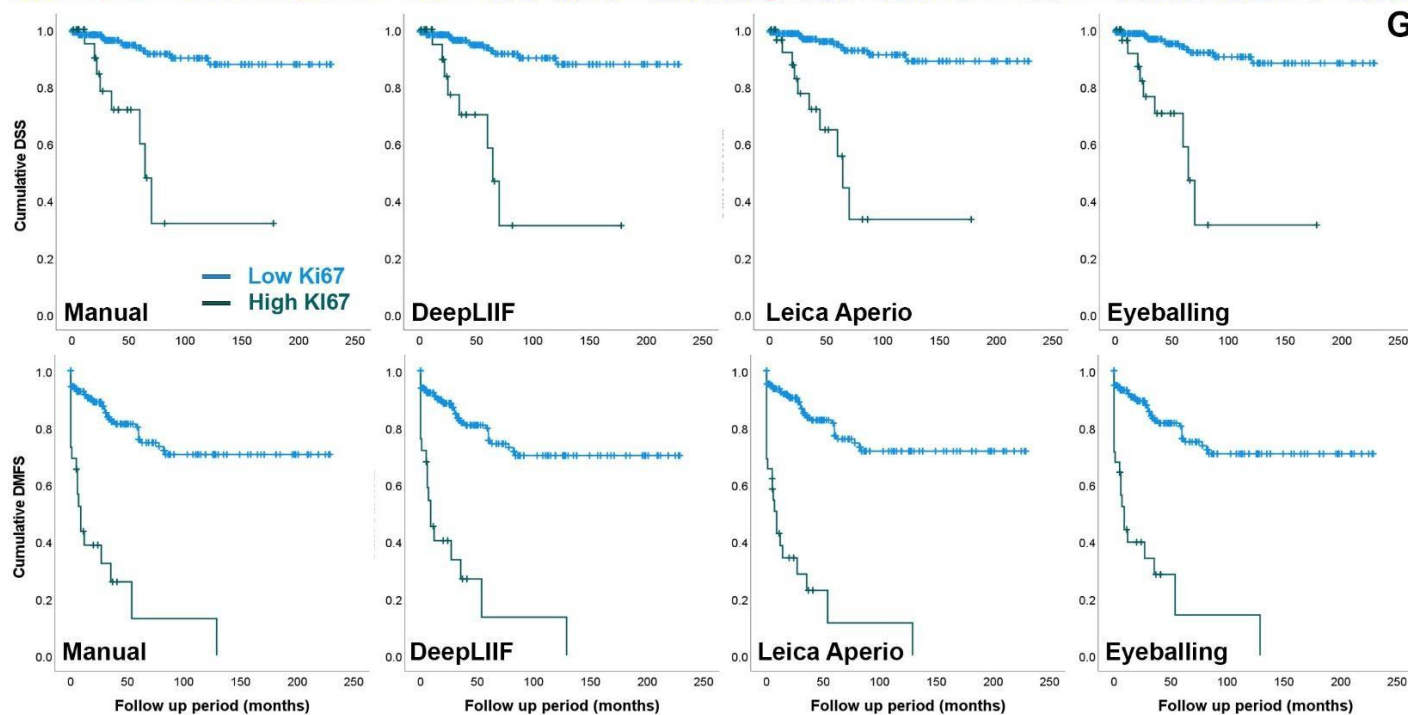
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D

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G

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