ABSTRACT

Background: Accurate diagnosis of melanoma remains histologically challenging. Dermal mitoses support malignancy, but are only occasionally seen in melanomas. Since melanomagenesis is thought to begin at the dermal-epidermal junction, we investigated the significance of epidermal melanocytic mitoses (EM) in a spectrum of lesions with molecular characterization.

Methods: EM density (EMD) was evaluated in 46 straightforward lesions (24 benign, 22 malignant) and 30 challenging lesions with expert interpretation, fluorescence-in-situ-hybridization, and myPath-score characterization (12 favor-benign, 9 favor-malignant, 9 ambiguous). EMD was correlated with clinicopathologic parameters and myPath.

Results: In straightforward cases, 25% nevi and 77% melanomas had EM. Median EMD was significantly lower in nevi vs. melanomas (0/mm vs 0.04/mm, p=0.001). EMD (0.01/mm-cutoff) had 77% sensitivity, 79% specificity discriminating melanomas from nevi. In challenging cases, 17% favor-benign, 67% favor-malignant, and 78% ambiguous lesions had EM. EMD (0.01/mm-cutoff) had 67% sensitivity, 82% specificity on 21 non-ambiguous lesions, similar to myPath. EMD was less accurate in Spitzoid lesions, which have high EMD and dermal mitoses.

Conclusions: While EMD is not an adequate single criterion in diagnosing melanoma, our results validate its discriminatory potential, suggesting that EM should prompt closer investigation for malignancy. Expanded studies with clinical follow-up are warranted to further assess the EM utility in classifying melanocytic lesions.

INTRODUCTION

Melanoma is the most life threatening form of skin cancer (1), yet it can be the most difficult to diagnose on histopathologic parameters (2-6). There are various morphologic mimickers of melanoma including nevi with high grade dysplasia and Spitz tumors (7, 8), and melanomas can occur in a broad age distribution (9). Thus, there exists a group of melanocytic proliferations in which definitive classification as benign or malignant either cannot be made with certainty, or requires the use of ancillary, including molecular, tests.

Criteria to distinguish melanoma have traditionally been divided into architectural and cytologic (10). Many of the architectural criteria used to diagnose melanoma - pagetoid spread of melanocytes, lentiginous growth, bridging of rete ridge nests, and misplaced/misshaped nests overlap to varying degrees with dysplastic nevi. Cytologic features used to classify melanoma such as cellular enlargement, prominent nucleoli, and reduced maturation can be seen in Spitz nevi. Dermal mitotic figures are somewhat rare (0-15% based on numerous studies) in benign nevi, and when combined with other histologic features of melanoma, are quite consistent with the diagnosis (11). This is related to the fact that most benign melanocytic lesions decrease proliferation and enter senescence once in the inhospitable environment of the dermis (12). Nevertheless, most early melanomas do not show dermal mitotic figures, hence this criterion may be of no aid in the discrimination of thin or borderline melanocytic proliferations.

Melanomagenesis takes place at the dermal epidermal junction (12), and yet the significance of epidermal mitotic figures in challenging melanocytic proliferations has not been systematically evaluated. Here we have assessed and quantified epidermal melanocytic mitoses in a cohort of histologically straightforward, and histologically difficult melanocytic lesions with molecular characterization (myPath gene expression score [Myriad Genetics] and fluorescence in-situ hybridization [FISH]) and expert consensus diagnosis. The presence and density of epidermal melanocytic mitoses was correlated with several clinicopathologic parameters and the myPath score.

MATERIALS AND METHODS

Following IRB approval, a total of 76 junctional or compound melanocytic lesions previously diagnosed at our institution, were evaluated for the presence of epidermal mitoses. The cases comprised 46 straightforward lesions and 30 challenging lesions (Table 1). The straightforward cases were diagnosed by the primary dermatopathologist on histologic basis only, and included 24 benign dysplastic nevi (16 moderate dysplasia, 8 severe dysplasia) and 22 melanomas (8 in situ, 12 superficial spreading melanoma, 1 lentigo maligna melanoma, 1 acral lentiginous melanoma). The challenging cases were diagnosed after expert consensus (JK, SDB, MT and AA) and analysis by melanoma-FISH using Vysis Melanoma FISH Probes (Abbott Molecular, Des Plaines, IL) for 6p25 (*RREB1*), CEP6, 6q23 (*MYB*), 11q13 (*CCND1*), 8q24 (*MYC*), 9p21 (*CDKN2A*) and CEP9 (centromeric reference). The final pathologic interpretation was favor benign in 12 cases (2 spitzoid, 1 non-dysplastic, 9 dysplastic), ambiguous in 9 cases (6 atypical Spitz tumor [AST], 3 atypical melanocytic proliferation [AMP]) and favor malignant in 9 cases.

All 46 straightforward cases and 28 of the 30 challenging cases were also characterized with myPath gene expression score (Myriad Genetic Laboratories, Salt Lake City, UT).

Epidermal melanocytic mitosis were identified and distinguished from keratinocyte mitoses based on the location within a melanocytic nest, the quality of the cytoplasm and the lack of cell adhesion structures. The epidermal mitoses were normalized for the amount of tissue present for evaluation as follows: the total number

of epidermal mitoses counted on all levels of all sections available for each lesion was divided by the number of levels and the measured junctional component length in millimeters (mm).The result expressed the density of epidermal mitoses /mm length for each lesion (epidermal mitotic density or EMD).

Other histologic parameters recorded were the Breslow depth and number of dermal melanocytic mitoses (for melanomas only) and the presence of associated inflammation, either in the form of chronic inflammatory infiltrates involving the melanocytic proliferations or as incidental chronic folliculitis. None of the lesions showed significant acute inflammation.

Statistical comparisons were made between the presence of epidermal mitoses or EMD and multiple clinicopathologic parameters including: patient age and sex, anatomic location of the lesion, histologic diagnosis, presence of inflammation, Breslow depth (for melanomas only) and myPath score. To derive p values, Fisher's exact test and chi-square test were used for categorical data, whereas Wilcoxon rank sum test and Kruskal-Wallis test were used for measured data.

RESULTS

Epidermal mitoses in straightforward dysplastic nevi and melanomas

Clinicopathologic features and mitotic prevalence

The clinicopathologic characteristics of the 46 straightforward cases are listed in Supplementary Table 1 and summarized in Table 2. Representative images of a dysplastic nevus and a melanoma with epidermal melanocytic mitoses are shown in Figure 1. Statistically significant differences in clinical parameters between patients with benign vs. malignant lesions included median age (51 vs. 61, p= 0.02) and frequency lesions on extremities (46% vs. 18%, p= 0.02). There was no significant difference in sex or presence of inflammation (Table 2).

Of 24 benign dysplastic nevi, 6 (25%) nevi (5 moderate dysplasia, 1 severe dysplasia) showed 1 or more epidermal mitoses (median 2, range 1-5) with a median EMD of 0/mm (range 0-0.10/mm). No dermal mitoses were seen. There was no significant difference in patient age or sex for dysplastic nevi with epidermal mitoses (median age 48.5; M:F=1:1) and nevi without epidermal mitoses (median age 52, p=0.56; M:F=2:1, p=0.63). There was also no difference in the anatomic location of the nevi with epidermal mitoses (trunk 3/6, 50%; leg 2/6, 33%; arm 1/6, 17%) and without epidermal mitoses (trunk 10/18, 56%; leg 4/18, 22%; arm 4/18, 22%). Chronic inflammation was present in 4/6 (67%) nevi with epidermal mitoses and in 10/18 (56%) nevi without mitoses (p=0.5).

Of 22 melanomas, 17 (77%) showed 1 or more epidermal mitoses (median 2, range 1-24) with a median EMD of 0.04/mm (range 0-0.32/mm), significantly higher than that in benign nevi (p=0.001) (Figure 2a). 5 melanomas showed no epidermal mitoses (3 in situ, 1 LMM, 1 SSM). Of the 14 invasive melanomas, only 4 had dermal mitoses (median 1.5/mm^2, range 1-5/mm^2), while 12/14 had epidermal mitoses (median 0.06/mm, range 0.02-0.32/mm). Interestingly, the epidermal mitotic density (mitoses/mm) showed a good correlation with the Breslow's thickness (mm) for all invasive melanomas (r=0.79; Figure 2b). There was no significant difference in patient age for melanomas with epidermal mitoses (median age 59) and melanomas without epidermal mitoses (median age 76; p=0.37). Although all 5 melanomas without epidermal mitoses were in men, the sample size was too small for a meaningful comparison. There was also no difference in the anatomic location of melanomas with epidermal mitoses (trunk 9/17, 53%; head/neck 4/17, 23%; arm 2/17, 12%; leg 1/17, 6%; acral 1/17, 6%) and without epidermal mitoses (trunk 4/5, 80%; head 1/5, 20%). Chronic inflammation or folliculitis was present in 12/17 (71%) of melanomas with epidermal mitoses and in 3/5 (60%) melanomas without mitoses (no significant difference, p=1).

Discriminatory power of epidermal mitoses

By applying a recursive portioning algorithm, an EMD cutoff of 0.01 mitoses/mm was calculated to best separate benign and malignant lesions. With this cutoff, EMD shows a sensitivity of 77% and a specificity of 79% in distinguishing straightforward

melanomas from nevi (Figure 2a, Table 2). Phenotypic characterization by myPath gene expression score was available on all of the cases in this cohort, as a discriminatory standard. Here, the gene expression score was concordant with the histologic diagnosis in 16/22 melanomas (73% sensitivity) and 19/24 dysplastic nevi (79% specificity) (Table 2). It was indeterminate in 5/24 (21%) nevi and 4/22 (18%) melanomas and discordant in 2/22 (9%) melanomas. The myPath score showed a weak correlation with the Breslow's thickness (r=0.23) on 14 invasive melanomas. It did not show a significant correlation with the patient age, sex, body site or presence of inflammation. Overall, for the straightforward lesions, EMD (cutoff 0.01) was non-inferior to myPath in distinguishing benign from malignant lesions. When using a combined test approach, where a "positive" result was considered if either EMD was >0.01 or myPath was positive in any combination with the other test's result, the sensitivity in detecting melanomas increased to 95% with 79% specificity.

Epidermal mitoses in challenging/borderline melanocytic proliferations

Clinicopathologic features and mitotic prevalence

The clinicopathologic characteristics of the 30 challenging cases are listed in Supplementary Table 2 and summarized in Table 3. Representative images of a Spitz nevus and an atypical Spitz tumor with epidermal melanocytic mitoses are shown in Figure 3. The sole baseline clinicopathologic feature which differed significantly between the favor-benign, ambiguous and favor-malignant lesions was the presence of inflammation, which was present in increasing proportions with increasing histologic

grade (17%, 56%, 89% respectively; p=0.004 overall, p=0.002 between favor-benign and favor-malignant). There was no significant difference in patient age, sex or anatomic location (Table 3). All favor-malignant lesions had molecular confirmation with positive melanoma-FISH, whereas all favor-benign and ambiguous lesions were FISH-negative. FISH results were known to the expert pathologists at the time of consensus diagnosis.

Epidermal mitoses were present in 2/12 (17%) favor-benign lesions (1/10 non-Spitz, 1/2 Spitz; median EMD 0/mm, range 0-0.75/mm), 7/9 ambiguous lesions (5/6 AST, 2/3 AMP; median EMD 0.11/mm, range 0-1.10/mm), and 6/9 (67%) favor-malignant lesions (median EMD 0.04/mm, range 0-0.4/mm) (Figure 4a). The difference in median EMD between the 3 groups of lesions was statistically significant (p=0.03). When isolated as a separate group, Spitz lesions (favor-benign and ambiguous AST) had by far the highest EMD (median 0.14/mm, range 0-1.1/mm) compared to the remaining favor-benign non-Spitz lesions (median 0/mm, range 0-0.11/mm, similar to that seen in the benign straightforward lesions), ambiguous AMP lesions (median 0.04/mm, range 0-0.04/mm) and favor-malignant lesions (median 0.04/mm, range 0-0.4/mm) (Figure 4b). Dermal mitoses were also present in 1/2 Spitz nevi and in 5/6 AST. In comparison, only 2/9, (22%) favor malignant lesions had dermal mitoses (median 2/mm^2). In this group of 9 favor-malignant lesions, EMD showed weak correlation with Breslow's thickness (Figure 4c, r=0.43) compared to that seen with straightforward melanomas.

Including all 30 challenging lesions, there was no significant difference in patient age or sex between those with epidermal mitoses (mean age 51; M:F=1:1.1) and those without epidermal mitoses (mean age 41, p=0.24; M:F=2:1, p=0.46). There was also no difference in the anatomic location of lesions with epidermal mitoses (trunk 9/15, 60%; arm 3/15, 20%; leg 2/15, 13%; head/neck 1/15, 7%) and without epidermal mitoses (trunk 8/15, 53%; leg 4/15, 27%; arm 1/15, 7%; head/neck 1/15, 7%; acral 1/15, 7%). (Table 3).

Discriminatory power of epidermal mitoses

EMD with a cutoff of 0.01/mm showed a sensitivity of 67% and a specificity of 82% in distinguishing the 9 favor-malignant lesions from the 12 favor-benign lesions (Table 3; Figure 4a). As a comparative standard, myPath gene expression scoring was concordant with the histologic diagnosis in 6/9 favor-malignant lesions (67% sensitivity) and 5/11 favor-benign lesions (45% specificity) (Table 3). In the ambiguous subgroup of challenging lesions, EMD would label 2/9 (22%) as benign (1 AST, 1 AMP) and 7/9 (78%) as malignant (5 AST, 2 AMP) based upon the cutoff of 0.01/mm. myPath categorized 4/8 (50%) lesions (3 AST, 1 AMP) as benign, 2/8 (25%) lesions (1 AST, 1 AMP) as indeterminate, and 2/8 (25%) lesions (1 AST, 1 AMP) as malignant. For one lesion, the test was technically unsuccessful. While true classification of these cases may be aided by longer term follow-up, overall, patients in this category would be expected to have excellent survival given that 6/9 cases represent FISH-negative (including 9p21) AST, and the remaining 3/9 cases of AMP would have Breslow's

thicknesses of (0.3, 0.5 and 0.6 mm). Overall, excluding ambiguous lesions, EMD (cutoff 0.01) was again non-inferior to myPath in distinguishing favor-benign from favor-malignant lesions. When using the same combined EMD-myPath test approach as described above, the sensitivity in segregating non-ambiguous lesions increased to 78% with 73% specificity.

DISCUSSION

Despite the repeated investigation of dermal mitoses in benign and malignant melanocytic proliferations (11, 13-17), relatively little information exists in the literature as to the prevalence, frequency and meaning of junctional mitoses in melanocytic lesions. Indeed, in our experience, junctional mitoses may be equally rare in typical banal nevi as compared to dermal mitoses. We inferred that epidermal mitoses may be biologically significant in melanoma and borderline lesions. Because melanomagenesis begins at the dermal epidermal junction, as manifest by the propensity of radial growth phase to precede vertical growth phase, this histologic sign may be more readily available in borderline cases. Mechanistically, the direct contact of melanoma cells with upper layer keratinocytes resulting from radial growth, was shown to instigate molecular interactions that trigger vertical invasion by melanocytes (18). Indeed, many of the genes recently associated with prognosis in melanoma represent keratinocyte genes, which may allude to an under recognized importance of the epidermal microenvironment (19). As such, we hypothesized that the presence and number/density of epidermal mitoses may represent another diagnostic clue in the assessment of borderline melanocytic lesions.

Our findings show that epidermal mitoses are more common and of higher density in straightforward malignant lesions as well as challenging cases that are ultimately designated as malignant compared to those straight away or ultimately classified as benign. This difference in prevalence (77% vs 25%, p=0.0012 in

straightforward; 67% vs 17%, p=0.059 in challenging) and median EMD (0 vs 0.04, p=0.001 in straightforward; 0 vs 0.04, p=0.06 in challenging) appears to be slightly stronger in straightforward lesions, as would be expected (Tables 2 and 3). Our studies confirm our impression that even *epidermal* mitoses are not found in the majority of even moderately and severely dysplastic *nevi* (75% without EM), where no dermal mitoses were found. In contrast, the majority of melanomas had EM, while only 18% of melanomas had dermal mitoses, making this histologic feature relatively less useful (albeit more specific). In straightforward nevi and melanomas, the presence of EM did not correlate with any clinical factors, and there was no significant difference in the amount of chronic inflammation in lesions with and without epidermal mitoses. These data suggest that in neoplasms falling on the dysplasia melanoma continuum, EM result from tumor cell autonomous factors rather than external ones.

When looking at the cohort of 30 challenging lesions, even after FISH and myPath studies, 9/30 cases were given an expert consensus designation of ambiguous. Of the 21 remaining cases, EM were significantly increased in favor-malignant cases compared to favor-benign as described above. Meanwhile, only 2/9 (22%) cases ultimately called melanoma had dermal mitoses, rendering this feature less applicable. The baseline clinicopathologic features were comparable in all 30 cases, aside from histologic inflammation which was significantly increased in the ambiguous and favor-malignant lesions, compared to those ultimately called benign (Table 3).

It bears deliberate discussion that Spitz lesions confound the data with the largest number of epidermal *and* dermal mitoses overall; and, as with every other aspect of their analysis, should be treated as their own entity. Spitz lesions are increasingly proving to represent a genetically and biologically unique group, as manifest by their increased propensity to spread to regional lymph nodes but be otherwise non-lethal for the most part. Indeed, even dermal mitoses have been previously described in non-malignant Spitzoid lesions (7) and were noted in our study as well. Our data support the tradition of allowing dermal mitoses in non-malignant Spitz tumors, and show the same for epidermal mitoses.

While expert histologic diagnosis still remains the gold standard for classifying melanocytic lesions (20), the inter-observer disagreement rate is non-trivial (21-23) and a subset of cases are still interpreted as ambiguous. Thus objective molecular tests like fluorescence in situ hybridization for melanoma-associated genomic aberrations or myPath gene expression score are being developed to improve diagnostic accuracy. The melanoma-FISH assay is better established (24), but not perfect. It does not show a 100% correlation with histologic diagnosis, but the pathologist faced with a challenging melanocytic lesion is hard-pressed not to interpret it as melanoma in the presence of a positive FISH result. On the other hand, since the assay covers only select genomic loci, malignancy cannot be excluded with a negative FISH result, but becomes less likely. Concordantly, in our study, all the lesions in the challenging group that were positive by melanoma-FISH were interpreted as favor-malignant. The myPath

gene expression score assay also distinguishes between benign and malignant melanocytic lesions with reported sensitivity and specificity comparable to those of FISH (25). As a difference, myPath has the caveat of an “indeterminate” result category for a small but significant fraction of cases. Furthermore, our recent work revealed discordant results between the two ancillary tests in a subset of cases, especially challenging lesions, for which unequivocal molecular results would be most needed (26).

We determined based on the straightforward group of cases that an EMD cutoff of 0.01/mm provides the best separation between benign and malignant lesions, with a sensitivity and specificity non-inferior to the myPath gene expression score. When applying this cutoff to the challenging group of cases, the sensitivity and specificity were only marginally lower than in the straightforward group, and remained non-inferior to the myPath score. While the overlap between the presence or absence of epidermal mitoses and a positive or negative myPath score was not complete, the gene expression score results provides additional validation for the potential discriminatory power of EMD between malignant and benign lesions. It is indeed possible that the myPath test includes a metric of genes expressed during junctional melanocyte proliferation.

In the challenging group, there was an interesting and significant increase in the proportion of cases with inflammation from favor-benign, to ambiguous and to favor-malignant lesions (Table 3). Inflammation may be regarded as a response to an aggressive melanocytic proliferation or mutation-associated neoantigen expression.

Conversely, it can be argued that inflammation may induce secondary melanocyte activation and proliferation, resulting in increased mitoses, possibly a modified molecular milieu and even skewing of a gene expression based test like myPath (26). Our results suggest that in straightforward cases, inflammation is not confounding and does not impact EM, but in borderline lesions inflammation can confound interpretation. The mechanisms by which inflammation influences histologic and molecular melanocytic attributes requires further study.

Our results additionally revealed a positive correlation between EMD and Breslow thickness in invasive melanomas, which was stronger in straightforward lesions. This is not explained simply by the overall size of the lesion, as EMD is independent of lesion breadth, but is more likely related to the biologic potential of the malignancy, where an aggressive tumor has an increased density of epidermal mitoses associated with a deeper dermal infiltration.

Evaluation of melanocytic lesions does not traditionally include an assessment of epidermal mitoses. Here we show that the presence of epidermal mitoses and EMD above 0.01/mm correlate with a diagnosis of malignancy and with the results of a molecular gene expression-based test. While calculating a standard EMD may be viewed as impractical and evaluation for epidermal melanocytic mitoses is not adequate as a single criterion in classifying melanocytic lesions, our study suggests that the presence of more than rare epidermal melanocytic mitoses should be considered as favoring a malignant diagnosis, and should prompt closer investigation for malignancy.

Further studies on an expanded cohort with clinical follow-up are warranted to further assess the diagnostic utility and biologic significance of epidermal melanocytic mitoses.

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LEGENDS

Table 1. Case distribution based on histopathologic interpretation (SSM – superficial spreading melanoma; LMM – lentigo maligna melanoma; ALM – acral lentiginous melanoma; AST – atypical Spitz tumor; AMP – atypical melanocytic proliferation).

Table 2. Statistical analysis of clinicopathologic features in the straightforward case group.

Table 3. Statistical analysis of clinicopathologic features in the challenging case group.

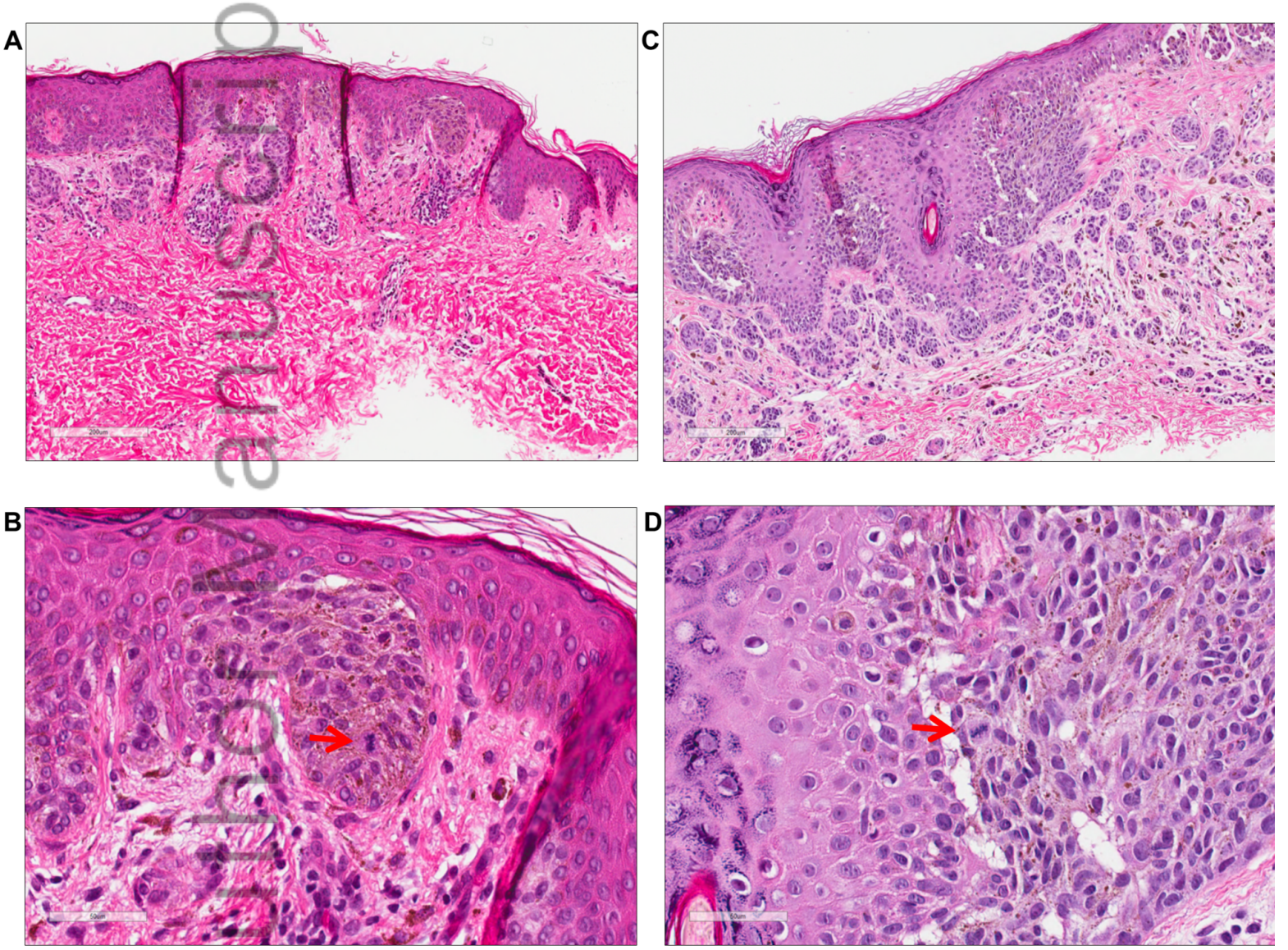
Figure 1. Representative images of unequivocal benign and malignant lesions with epidermal melanocytic mitoses (arrows): dysplastic nevus (A. 100x, B. 400x); melanoma (C. 100x, D. 400x).

Figure 2. Distribution of EMD in straightforward benign and malignant lesions (A) with a cutoff of 0.01/mm (median EMD on top). Correlation between EMD and Breslow thickness on 14 straightforward invasive melanomas (B) (r_S Spearman non-parametric coefficient).

Figure 3. Representative images of challenging lesions with epidermal melanocytic mitoses (arrows): Spitz nevus (A. 100x, B. 400x); atypical Spitz tumor (C. 100x, D. 400x).

Figure 4. Distribution of EMD in challenging favor-benign, ambiguous and favor-malignant lesions (A) with a cutoff of 0.01/mm (median EMD on top). Similar distribution

of EMD when Spitzoid lesions (favor-benign and ambiguous) are grouped separately (B). Correlation between EMD and Breslow thickness on 9 invasive favor-malignant lesions (C) (r_S Spearman non-parametric coefficient).



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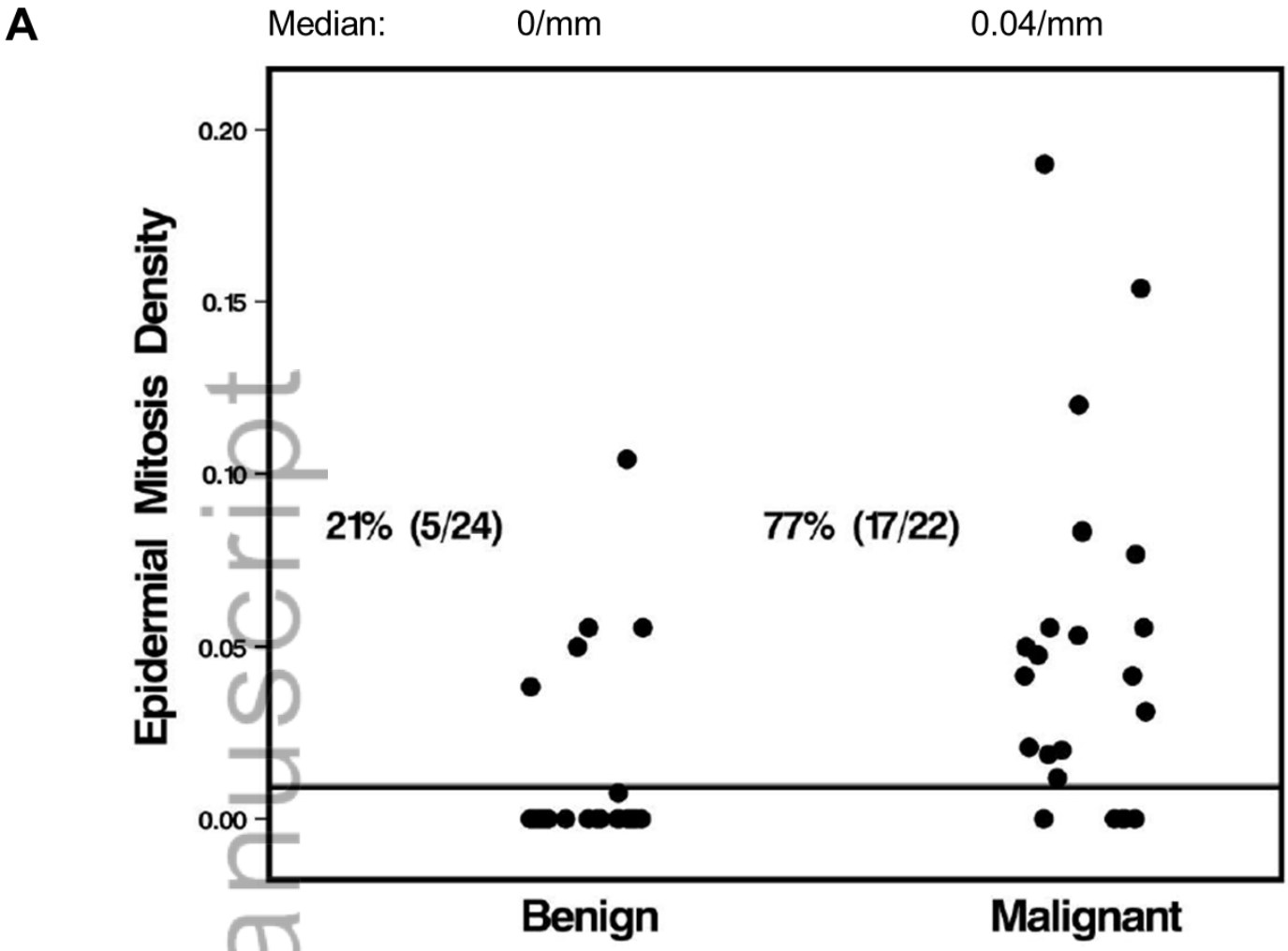

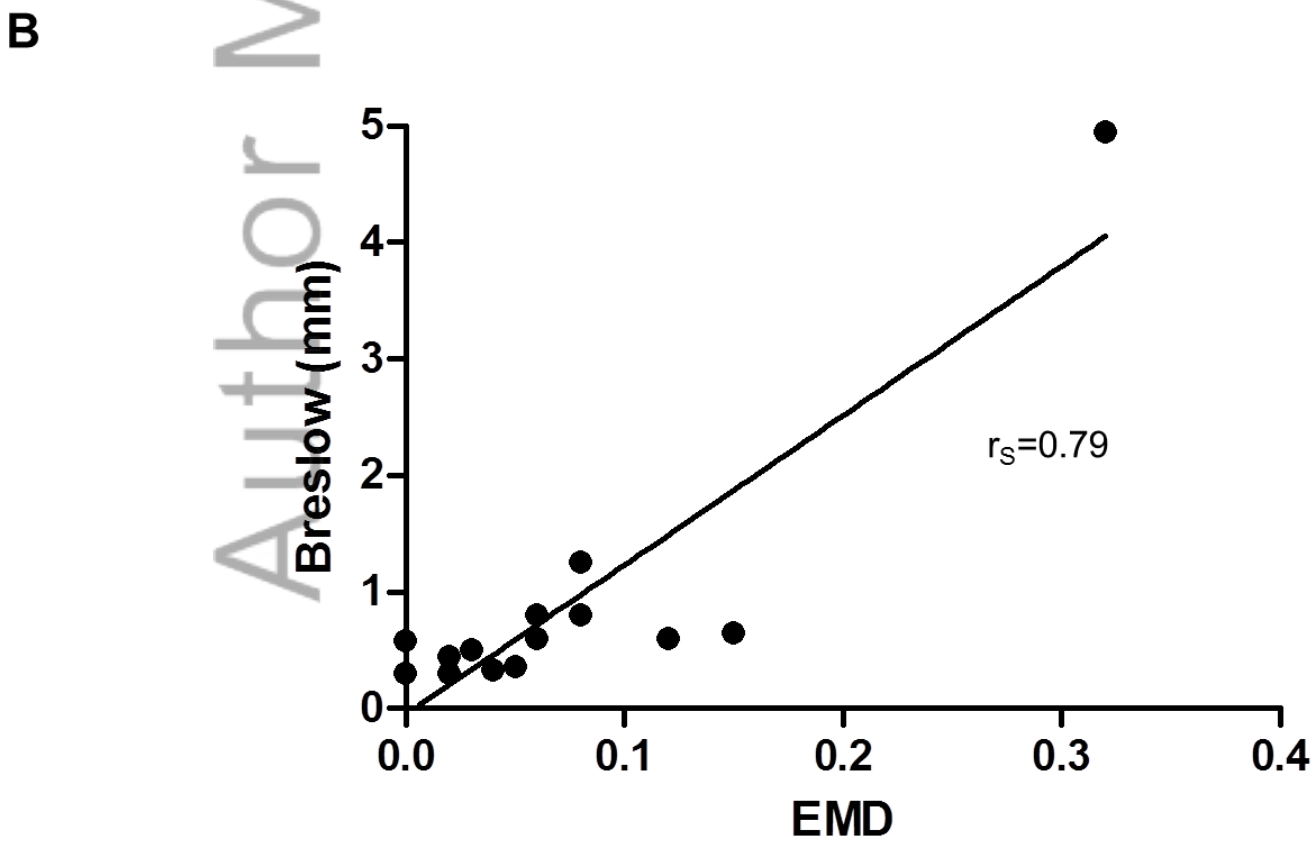

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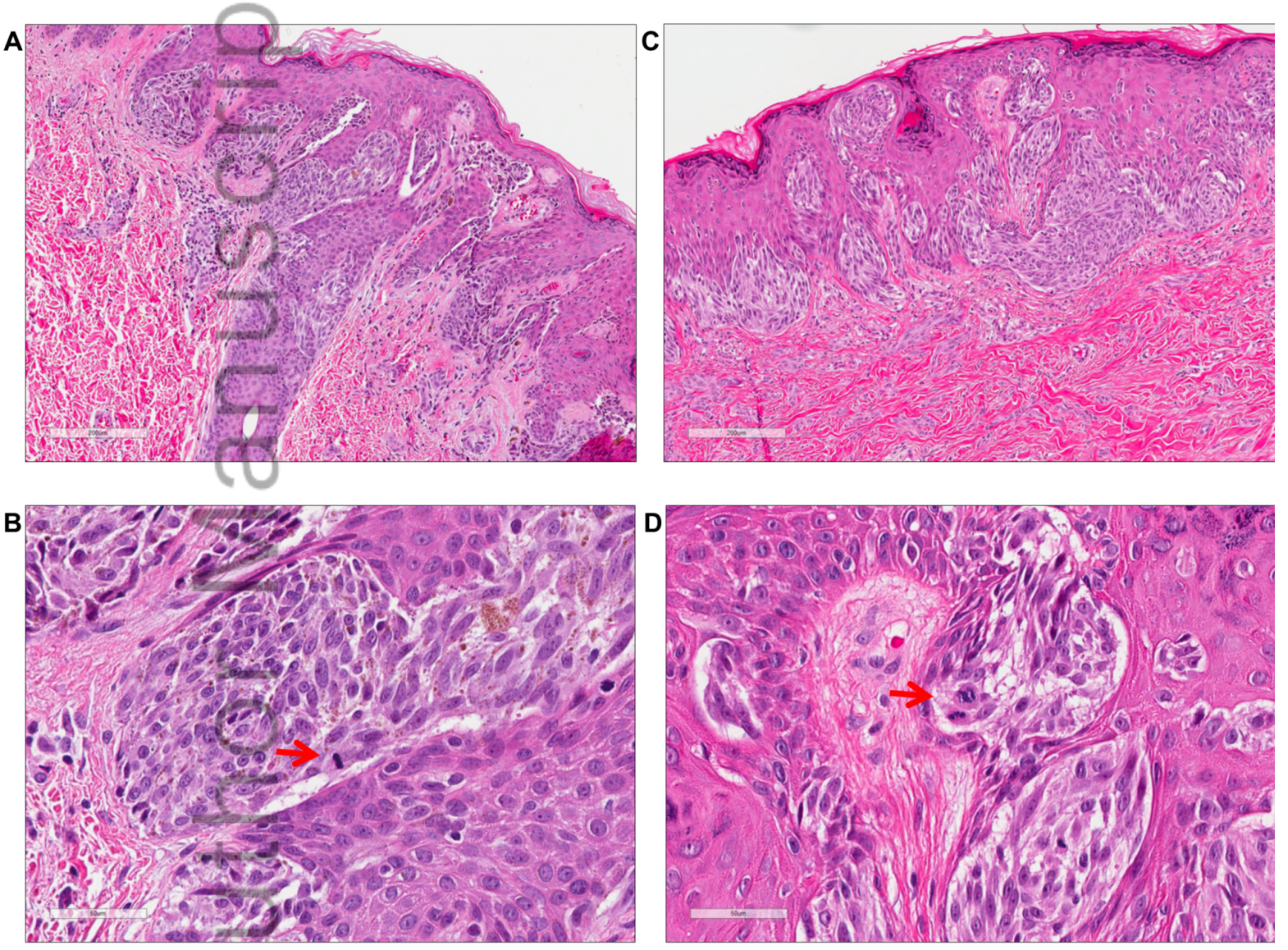

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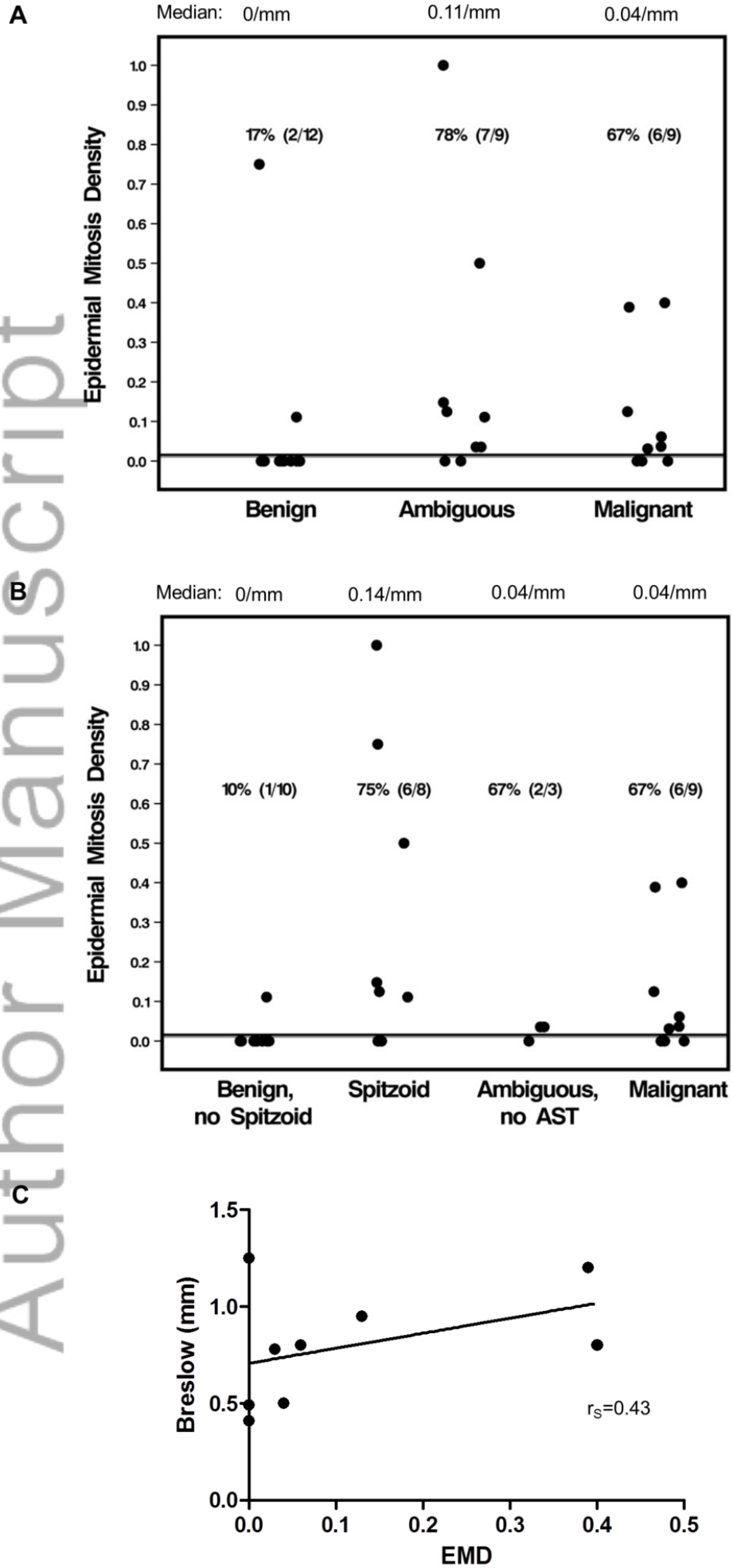

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Significance of Epidermal Mitoses in Challenging Melanocytic Proliferations

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