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# Epidermal AMBRA1 and Loricrin; a paradigm shift in the prognostication and stratification of AJCC stage I melanomas

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**Conflicts of Interest**

Dr Rob Ellis, Dr Marie Labus and Professor Penny Lovat are directors of AMLo Biosciences Ltd.

## Translational relevance

### What is already known?

- There is an unmet clinical need for biomarkers of early stage melanoma
- AMBRA1 is a pro-autophagy regulatory protein with known roles in cell proliferation and differentiation; and is a known tumour suppressor.
- Loricrin is a marker of epidermal terminal differentiation

### What does the study add?

- AMBRA1 has a functional role in keratinocyte/epidermal proliferation and differentiation
- The combined decrease/loss of peri-tumoural AMBRA1 and Loricrin is associated with a significant increased risk of metastatic spread in AJCC stage I tumours versus melanomas in which peri-tumoural AMBRA1 and Loricrin are maintained, independent of Breslow depth.

### What is the translational message?

The adoption of peri-tumoural epidermal AMBRA1/Loricrin biomarker expression into melanoma care guidelines will facilitate more accurate, personalised risk-stratification for patients with AJCC I melanomas, thereby facilitating stratification for appropriate follow-up and informing on post diagnostic investigations including SLNB, ultimately resulting in improved disease outcomes and rationalisation of healthcare costs.

**Summary**

**Background**

Despite the recent update to the AJCC staging criteria for melanoma, this system is still unable to identify truly high-risk stage I tumour subsets.

**Objective**

To determine clinical utility of combined epidermal AMBRA1/Loricrin (AMLo) expression as a prognostic biomarker for AJCC stage I cutaneous melanoma.

**Methods**

Peri-tumoural AMBRA1 expression was evaluated in a retrospective discovery cohort of 76 AJCC I melanomas. Multivariate analysis of AMLo expression was subsequently correlated with clinical outcomes up to 12-years in two independent powered, retrospective validation and qualification cohorts comprising of 379 AJCC I melanomas.

**Results**

Decreased AMBRA1 expression in the epidermis overlying primary melanomas in a discovery cohort of 76 AJCC I tumours was associated with 81.5% 7-year DFS versus 100% survival with maintained AMBRA1; P<0.081. Following automated IHC protocol development for semi-quantitative analysis of AMLo further analysis was undertaken in validation (n=218) and qualification cohorts (n=161) of AJCC I melanomas. Combined cohort analysis revealed a DFS of 98.3% in the AMLo low-risk group (n=239) versus 85.45% in the AMLo high-risk cohort (n=140, P<0.001). Sub-cohort, multivariate analysis revealed the AMLo hazard ratio of 4.04 ((95% CI 1.69-9.66) P = 0.002), is a stronger predictor of DFS than Breslow depth (multivariate analysis 2.97 (95% CI 0.93-9.56) P = 0.068) in AJCC stage IB patients.

**Conclusions**

Loss of AMLo expression in the epidermis overlying primary AJCC stage I melanomas identifies high risk tumour subsets independently of Breslow depth and represents a major paradigm shift in future prognostic assessment and stratification of primary melanomas.

**INTRODUCTION**

Melanoma is one of the most devastating skin cancers, with a worldwide incidence that continues to climb (1), (2). Although the introduction of targeted and immune-therapies has revolutionised treatment of metastatic disease, the largest proportion of patients presenting to clinicians however, have thin, early stage melanomas yet to benefit from therapeutic innovation, in part related to a lack of credible biomarkers of disease progression.

Currently, disease staging and risk prediction is based on histological characterisation of the primary tumour; including depth of tumour invasion (Breslow depth) and the presence of epidermal ulceration, forming the basis of the 7$^{th}$ edition American Joint Committee on Cancer (AJCC) staging criteria (3). The recently updated 8$^{th}$ edition AJCC guidelines came into effect in January 2018 (4), with removal of mitotic count and reduction of Breslow depth for stage IA melanomas to 0.8mm. However, these criteria are still unable to reliably identify which individuals with seemingly low risk early melanomas are at specific risk of disease progression; occurring in up to 15% of patients with AJCC I melanoma (4, 5). An urgent unmet need for credible prognostic biomarkers able to identify patients with high risk early stage melanomas, facilitating appropriate counselling and follow up (including guidance on appropriate need for sentinel lymph node biopsy (SLNB)) or access to clinical trials and potentially adjuvant systemic treatment (6, 7), thus remains.

We have identified two protein markers, AMBRA1 and Loricrin, in the epidermis overlying primary melanomas, whose expression is lost in high-risk AJCC I melanomas, but which are retained over genuinely low-risk tumours. The role of AMBRA1 (a pro-autophagy regulatory protein) in melanoma progression was initially evaluated by immunohistochemistry (IHC) in a retrospective melanoma discovery cohort following previous reports of the role of autophagy in melanomagenesis (8, 9); however, unlike previously investigated autophagy biomarkers such as p62 (8), AMBRA1 revealed variations in expression within the epidermis overlying primary melanomas, rather than within the tumour itself, suggesting a specific role for this protein in epidermal differentiation (9). In the present

study to investigate peri-tumoural AMBRA1 as a potential prognostic marker, we initially evaluated immunohistochemical semi-quantitative expression in a retrospective discovery cohort of AJCC stage I melanomas. However, although loss of peri-tumoural AMBRA1 expression correlated with disease progression with an assay sensitivity of 100%, the relatively low assay specificity (33%) hindered its clinical utility. Subsequently to improve specificity, peri-tumoural epidermal AMBRA1 expression was assessed in combination with epidermal Loricrin (as a marker of keratinocyte terminal differentiation), with the primary aim of this study to validate combined peri-tumoural AMBRA1 and Loricrin (AMLo) expression as a prognostic biomarker for early stage melanoma.

## MATERIALS AND METHODS

### Study design

### Patient cohort selection

This study included three, independent, statistically powered retrospective cohorts of AJCC stage I melanomas defined by the AJCC 7th edition (figure 1). All cohorts were accessed following ethical permission (REC Ref 08/H0906/95+5_Lovat). All steps of biomarker development followed the Cancer Research UK Prognostic Biomarkers Roadmap (10) and reported in line with REMARK guidelines (11). The initial discovery cohort of 76 patients was derived from Newcastle Hospitals NHS Foundation Trust (NuTH), with subsequent discovery stage 2 (validation) and qualification retrospective cohorts derived from James Cook University Hospital (JCUH; n=218) and University Hospital of North Durham respectively (UHND; n=161). Full patient selection and cohort demographics are described in supplementary figure 1.

### Semi-quantitative IHC analysis of AMBRA1 and Loricrin

5 µm formalin-fixed, paraffin-embedded (FFPE) tissue sections were derived from primary melanomas in each cohort. IHC methodology for AMBRA1 (Abcam), Loricrin (Abcam) and cytokeratin 5 (Novocastra) are detailed in the supplementary methods.

Semi-quantative analysis of epidermal AMBRA1 expression was undertaken using Leica Digital Image Hub software (Leica Biosystems). Up to ten representative x200 microscope fields were analysed for mean positive pixel intensity of AMBRA1 expression levels and compared to the mean AMBRA1 expression in the epidermis directly above the melanoma allowing a relative percentage expression change to be calculated with the normal epidermis considered as 100% expression.

**Validation of AMBRA1 and Loricrin Scoring**

Visual AMBRA1 and Loricrin scoring for all cohorts was undertaken by three individuals blinded to outcomes (RAE, EC, GW); consensus agreement was reached for all samples. The "by-eye" analysis was grouped as either "Maintained AMBRA1" or "Decreased/lost AMBRA1". "Maintained AMBRA1" was defined as no discernible difference in AMBRA1 expression between normal and peri-tumoural epidermis. "Decrease/lost AMBRA1" was defined as *any* decrease or complete loss of AMBRA1 expression between normal and peri-tumoural epidermis.

All loricrin expression analysis was undertaken by eye, and loss of expression defined as any discernible break in the continuity of expression in the *stratum corneum*.

**Statistical Analysis**

Survival analyses were conducted using the R function coxph(). Univariate estimates presented are coefficients, with 95% confidence estimates, resulting from a Cox fit with only that covariate as a predictor, while multivariate estimates are the associated coefficient from a full additive model including all reported covariates (in particular, we did not re-fit to exclude non-significant predictors and considered no interaction terms).

Survival curves were generated using the R function survfit() to present Kaplan-Meier curves for a univariate model based on AMBRA1/Loricrin status, with presented P values as the result from a Log Rank (score) test for the associated univariate Cox model.

Statistical analysis for disease free survival (DFS) was planned for each cohort independently, as well as in combination for the James Cook University Hospital (JCUH) and the University hospital of North Durham (UHND) cohorts. Further sub-cohort analysis was undertaken in AJCC stage IA and IB patients,

as well as patients eligible for sentinel lymph node biopsy (SLNB) under the current UK National Institute for Health and Care Excellence (NICE) i.e with a Breslow depth >1mm.

For the analysis of SLNB data sets, post-test odds are used to calculate the positive and negative predictive values (i.e. clinically relevant measures of diagnostic accuracy). These are calculated by multiplying the pre-test probability of a positive (negative) result (the prevalence or 1 – prevalence) with the diagnostic likelihood ratio of a positive (negative) test (12).

## RESULTS

### Peri-tumoral AMBRA1 expression and DFS in the NUTH Discovery cohort

To identify an association between epidermal AMBRA1 expression overlying primary tumours (peri-tumoural AMBRA1) and DFS in patients with AJCC I melanoma, 76 patients within the Newcastle Discovery cohort were stratified as either **maintained** or **decreased** AMBRA1 expression by visual analysis of peri-tumoural AMBRA1 expression (figure 2). All samples were also analysed for cytokeratin 5 (CK5; figure2A), a pan-epidermal marker, which revealed no association between the degree of melanoma epidermal invasion and peri-tumoural AMBRA1 expression.

Kaplan-Meier survival analysis revealed reduced 7-year DFS in patients with **decreased** peri-tumoural epidermal AMBRA1 expression compared to 22 patients with **maintained** AMBRA1 expression (81.5% vs 100%; P =0.081, figure 2B). The hazard ratio (HR) for disease recurrence among patients with **decreased** AMBRA1 expression versus **maintained** expression however, could not be assessed due to a lack of events in the **maintained** cohort.

Although **decreased** AMBRA1 expression was associated with a reduced 7-year DFS with a sensitivity for identifying patients at risk of disease progression of 100%, the specificity of the test was only 33.3% (figure 2D), limiting clinical utility.

### AMBRA1 as a marker of epidermal differentiation

To further underpin the role of AMBRA1 in epidermal differentiation, western blot analysis for the expression of AMBRA1 and associated epidermal proteins was performed in primary keratinocytes undergoing calcium-induced differentiation in vitro. Results revealed a consistent time-dependent increase in AMBRA1 expression in line with increased differentiation; highlighted by decreased

expression of CK14 (a marker of basal keratinocytes) and increased loricrin expression. Conversely, siRNA-mediated knockdown of AMBRA1 in primary keratinocytes resulted in deregulated differentiation as evidenced by down regulation of loricrin at both the mRNA and protein level (supplementary figure 2).

**Combined AMBRA1/Loricrin peri-tumoural epidermal expression and DFS in the JCUH and UHND validation cohorts**

To compare the method of determining AMBRA1 expression, both visual and semi-quantitative analysis of peri-tumoural AMBRA1 expression were performed in the JCUH cohort (figure 3, supplementary figure 4). Results revealed a significant difference in median percentage loss of AMBRA1 expression from 11.2% in the "no visual loss" group to 84.1% in the "visual loss present" group (figure 3B; Mann-Whitney $P<0.001$). As such, any decrease or loss of peri-tumoural epidermal AMBRA1 expression when compared "by eye" with the expression of AMBRA1 in normal epidermis was deemed as "high-risk" (supplementary figure 4). Furthermore, these observations confirmed the robustness of visual analysis, which was subsequently used alone for all other cohort analyses, and highlighted the major benefit of having an internal control (normal epidermis) present in each primary tumour excision sample.

Visual assessment of Loricrin also defined two distinct sub-sets (figure 3B, supplementary figure 5).

To confirm the association of decreased DFS with alterations of the peri-tumoural epidermis, IHC expression of loricrin was also assessed in a small sub-group of the NUTH discovery cohort of AJCC stage I melanomas revealing an association between peri-tumoural loricrin loss and decreased DFS with a high degree of assay specificity but lower sensitivity (supplementary figure 6). Strikingly, however, when epidermal loricrin expression was combined with epidermal AMBRA1 expression, results revealed decreased peri-tumoural expression of both markers was associated with 100%

sensitivity and specificity for identifying truly high-risk melanomas in this sub-cohort (supplementary figure 6).

Consequently, the combination of AMBRA1/Loricrin was deemed as “high-risk” if AMBRA1 peri-tumoural expression was decreased or lost, *and* if there was any apparent break in the continuous expression of epidermal loricrin.

Subsequent analysis of combined epidermal AMBRA1 and loricrin expression was undertaken in two further validation cohorts of AJCC I melanoma patients (JCUH and UHND cohorts). These cohorts were statistically powered to provide 80% and ~95% power respectively to detect an HR of >4.0 (as per biomarker discovery cohort) at the P= 0.05 level, assuming equal group sizes and a representative number of metastatic events as expected in AJCC I melanomas (~10%) (3). A detailed description of patient data sets for these analyses is provided in supplementary figure 2.

The IHC protocol was further refined from the Discovery cohort as detailed in the supplementary methods, and undertaken on a fully automated clinical platform within the JCUH Pathology Department (figure 3B).

Analysis of 10-year DFS in the JCUH cohort of 218 AJCC I melanomas revealed reduced DFS in 60 patients stratified as AMBRA1/Loricrin high-risk compared to 158 patients defined as AMBRA1/Loricrin low-risk (83.3% vs. 98.7%; *P*=0.001, figure 4A) with a multivariate HR for disease recurrence among patients with high-risk AMBRA1/Loricrin expression of 7.28 (95% CI 2.36 – 22.4 13; P<0.001, figure 4B).

Analysis of DFS over 12 years in the 161 patients in the UHND cohort also demonstrated reduced DFS in 80 patients with high-risk AMBRA1/Loricrin expression compared to 81 patients with low-risk AMBRA1/Loricrin expression (88.8% vs 97.5%; *P*=0.03, figure 4C). The multivariate HR for disease recurrence among patients with high-risk AMBRA1/Loricrin expression in this cohort was 2.58 (95% CI 0.87-7.65; P=0.088, figure 4D).

By combining the two validation cohorts (figure 5) to increase the power to detect an effect of AMBRA1/Loricrin alone, results revealed a highly significant reduction in DFS in 140 patients with high-risk AMBRA1/Loricrin expression compared to 239 patients with low-risk AMBRA1/Loricrin expression (85.5% vs 98.3%; *P*<0.001, figure 5A). The multivariate HR for disease recurrence among patients with high-risk AMBRA1/Loricrin expression was 3.89 (95% CI 1.8-8.41; *P*<0.001, figure 5B). As a further guide to clinical utility, analysis of the combined cohort revealed a sensitivity of 82.6%, specificity of 66%, a Positive Predictive Value of 13.6%, but most importantly, a Negative Predictive Value of 98.3% (figure 5B).

Furthermore, sub-group analysis of the study combined cohort of stage IB ($8^{th}$ edition AJCC) patient set (figure 5C &D) revealed a statistically stronger correlation between AMBRA1/Loricrin stratification and DFS. In this sub-cohort, DFS was 97.1% in the AMBRA1/Loricrin low-risk group (n=105) versus only 79.6% in the AMBRA1/Loricrin high-risk cohort (n=97, $P < 0.001$, figure 5C). Multivariate analysis revealed an AMBRA1/Loricrin HR of 4.04 ((95% CI 1.69-9.66) P = 0.002, figure 5D), whereas there was less of a correlation between Breslow depth and overall DFS; multivariate analysis 2.97 ((95% CI 0.93-9.56) P=0.068, figure 5D), suggesting that AMBRA1/Loricrin is a prognostic marker, independent of Breslow depth, which is able to stratify patient risk over-and-above AJCC staging alone.

**DISCUSSION**

We are currently experiencing what has previously been described as the “golden age” of melanoma therapy (13), with an ever expanding arsenal of systemic medications available for patients with metastatic disease resulting in increased survival periods beyond the expectations of the most optimistic of clinicians even 10 years ago (14, 15). The thrust of trials is now aimed at adjuvant initiation of these therapies which has resulted in unprecedented results in patients with surgically resected, AJCC stage III melanoma at high risk of recurrence (16, 17). The natural progression to finally tackling melanoma is the initiation of systemic therapy at the earliest possible point at which high-risk individuals can be identified; thus stopping the development of life threatening metastases, or treating them before they have affected the patient’s health.

As such, a yet unrealized dream for the management of melanoma is the ability to identify patients at the highest risk of progression as close to the time of diagnosis of a primary melanoma as possible; allowing increased surveillance and earlier therapeutic intervention. Conversely, patients at truly low risk of disease could be more confidently reassured, and follow-up regimes altered. The largest population of patients affected by melanoma have AJCC stage I disease. As such, any improvements in the care of this group of patients will affect the largest number of patients overall, with the associated potential health economic benefits.

AJCC staging of melanoma alone, relying on Breslow depth and the presence of ulceration is not a perfect predictor of outcome in the lowest risk, stage I group; where a small but significant proportion of patients will still die of their disease. Recent, evidence based changes to the AJCC 8$^{th}$ edition staging criteria highlight a “breakpoint” of 0.8mm Breslow, with non-ulcerated primaries below this depth being classified as stage 1A, with ulcerated tumours <0.8mm, or non-ulcerated tumors from 0.8 - 2mm Breslow classified as stage IB. This has increased the number of patients now classified as stage IB,

with implications on increased surveillance for stage IB patients (5 years follow-up) versus IA (1 year follow-up).

In the present study we describe the discovery and validation of the combined expression of two protein biomarkers, AMBRA1 and loricrin (AMLo) in the epidermis overlying primary AJCC stage I melanomas as a highly sensitive and specific prognostic biomarker. AMBRA1 is a pro-autophagy regulatory protein and our *in vitro* data further define a functional role for AMBRA1 in epidermal proliferation, and like loricrin, in epidermal differentiation.

There is ongoing controversy about the clinical role of sentinel lymph node biopsy (SLNB) with large scale trials revealing questions about the prognostic and therapeutic role of SLNB, as well as highlighting the associated morbidity and healthcare costs of patients undergoing the procedure unnecessarily. As with melanoma in general, improved stratification of those patients at the highest risk of disease progression would potentially allow SLNB to further refine individual disease risk.
The MSLT-1 SLNB trial (18) contained a cohort of 765 intermediate thickness primary tumours undergoing SLNB that ranged from 1-3.5mm Breslow depth. Overall, the pre-test probability of a patient in this cohort developing a metastasis was 16%. In patients with a positive SLNB the number developing metastases was higher, giving a post-test probability of developing metastases in this group at 33% (95% CI 29-36%); however, a negative SLNB was still associated with a 13% chance of disease progression (95% CI 11-15%) (figure 6) (18). These data therefore suggest that a positive SLNB is able to further risk stratify patients, yet a negative SLNB adds little to reassure patients about their true risk of metastasis.

Although analysis has been undertaken in separate cohorts, assessment of the outcomes in our SLNB eligible combined cohort (patients with a Breslow depth over 1mm as per UK guidelines) nevertheless revealed a pre-test probability of metastasis of 10% is increased to 18% (95% CI 12 - 24%) in the

AMBRA1/Loricrin high-risk group. In contrast, low-risk AMBRA1/Loricrin however, was associated with a post-test probability of only 1.4% chance of metastasis (95% CI 0 - 5%, figure 6). In this context, these results highlight the potential of AMBRA1/Loricrin as a valuable pre-SLNB test; identifying those patients that would receive no further benefit from SLNB, and increasing the positive predictive value of SLNB through use in only a high risk, AMBRA1/Loricrin refined cohort.

In summary, our study reveals AMBRA1/Loricrin as a novel prognostic biomarker for early stage cutaneous melanoma, over-and-above AJCC staging alone. This simple, IHC-based marker will integrate seamlessly into standard clinical pathways of melanoma diagnostics, and allow a greater degree of certainty around disease outcomes for the treating clinician. Given the current prevailing view that SLNB is a purely prognostic tool, and considering the cost and morbidity associated with SLNB there is potential that the AMBRA1/loricrin biomarker may later replace SLNB. Not only will this benefit the individual patient in terms of reduced psychological burden from greater clarity of disease risk, but it will also allow better healthcare resource utilization internationally. As the golden age of melanoma care continues, the adoption of the AMBRA1/Loricrin biomarker into clinical guidelines thus presents a major paradigm shift in melanoma prognostication and stratified personalized management for the future.

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**FIGURE LEGENDS**

**Figure 1. Study Design.**

Study design followed the Cancer Research UK prognostic biomarker roadmap (10). Following initial identification of varied AMBRA1 epidermal expression in formalin-fixed, paraffin-embedded AJCC (2009) stage I and II primary cutaneous melanoma, an IHC assay was developed for initial assessment in a retrospective cohort of 76 AJCC stage I melanoma patients derived from Newcastle Hospital NHS Foundation Trust. Following validation and conversion of the assay to a fully automated IHC system,

and the addition of Loricrin, two further retrospective cohorts of 218 and 161 patients with AJCC (2009) stage I melanomas were analysed for AMBRA1/Loricrin expression levels and associated disease-free survival data.

**Figure 2. Relationship between peri-tumoural AMBRA1 expression and disease-free survival in the Newcastle Discovery AJCC stage I melanoma cohort.**

(A) Representative photomicrographs of immunohistochemical AMBRA1 (pink) or cytokeratin 5 (CK5; brown) staining in normal or matched peri-tumoural epidermis, where (e) represent the epidermis and (m) identifies melanoma (Breslow depth = 1.2 mm, scale bars = 100 μm). (B) AMBRA1 levels in the peritumoural epidermis of AJCC stage I melanomas were determined by pathologist visual inspection and defined as maintained or decreased. Estimated 7-year disease free survival rates were determined with the Kaplan-Meier method and compared by two-sided Log-Rank test (81.5% vs 100%; P<0.081). (C) Univariate and multivariate cox regression analysis of disease-free survival. (D) Performance of the AMBRA1 assay as defined by clinical sensitivity and specificity, and positive and negative prediction values.

**Figure 3. IHC analysis of AMBRA1 protein expression in the JCUH AJCC stage I cohort.**

(A) Semi-quantitative scoring of AMBRA1 (percentage decrease in peritumoural epidermis compared to normal epidermis at the section margins) versus visual scoring of either maintained or decreased peritumoural AMBRA1 (horizontal bar = median with IQR (Median = 11.2% vs 84.1%; Mann-Whitney P<0.001). (B) Representative photomicrographs of maintained (low risk) or decreased (High risk) immunohistochemical AMBRA1 staining in peri-tumoural epidermis at 100x and 200x magnification (scale bars = 100 μm). (c) Representative photomicrographs of continuous (low risk) or broken (High

risk) immunohistochemical loricrin staining in peri-tumoural epidermis at 100x and 200x magnification (scale bars = 100 µm).

**Figure 4. Relationship between peri-tumoural AMBRA1/Loricrin expression and disease-free survival in the validation cohorts.**

AMBRA1 and Loricrin levels in the peritumoural epidermis of AJCC stage I melanomas were determined by pathologist visual inspection and defined as maintained or decreased. 10-year disease free survival rates were determined with the Kaplan-Meier method and compared by two-sided Log-Rank test in both the JCUH (A; 84.8% vs 98.1%; P<0.001), and UHND (C; 88.8% vs 97.5%; P=0.033) cohorts. (B, D) Assay performance and univariate and multivariate cox regression analysis of disease-free survival in the JCUH (B) and UHND (D) cohorts.

**Figure 5. Relationship between peri-tumoural AMBRA1/Loricrin expression and disease-free survival in the combined validation cohorts.**

AMBRA1 and Loricrin levels in the peritumoural epidermis of AJCC stage I melanomas were determined by pathologist visual inspection and defined as maintained or decreased. 12-year disease free survival rates were determined with the Kaplan-Meier method and compared by two-sided Log-Rank test in the combined validation cohort (86.1% vs 97.9%; P<0.001). (B) Assay performance and univariate and multivariate cox regression analysis of disease-free survival in the combined cohort. (C) 12-year disease free survival rates were determined with the Kaplan-Meier method and compared by two-sided Log-Rank test in AJCC Stage IB melanomas of the combined validation cohort (80.5% vs. 96.2%; P<0.001). (D) Assay performance and univariate and multivariate cox regression analysis of disease-free survival in AJCC Stage IB melanomas of the combined cohort.

**Figure 6. Relationship between peri-tumoural AMBRA1/Loricrin expression and disease-free survival in SLNB eligible patients.**

(A) Post-test probabilities after SLNB for metastatic melanoma in MSLT1 intermediate thickness cohort. (B) Post-test probabilities after analysis of AMBRA1/Loricrin expression in SLNB eligible AJCC I melanoma samples.

Figure1

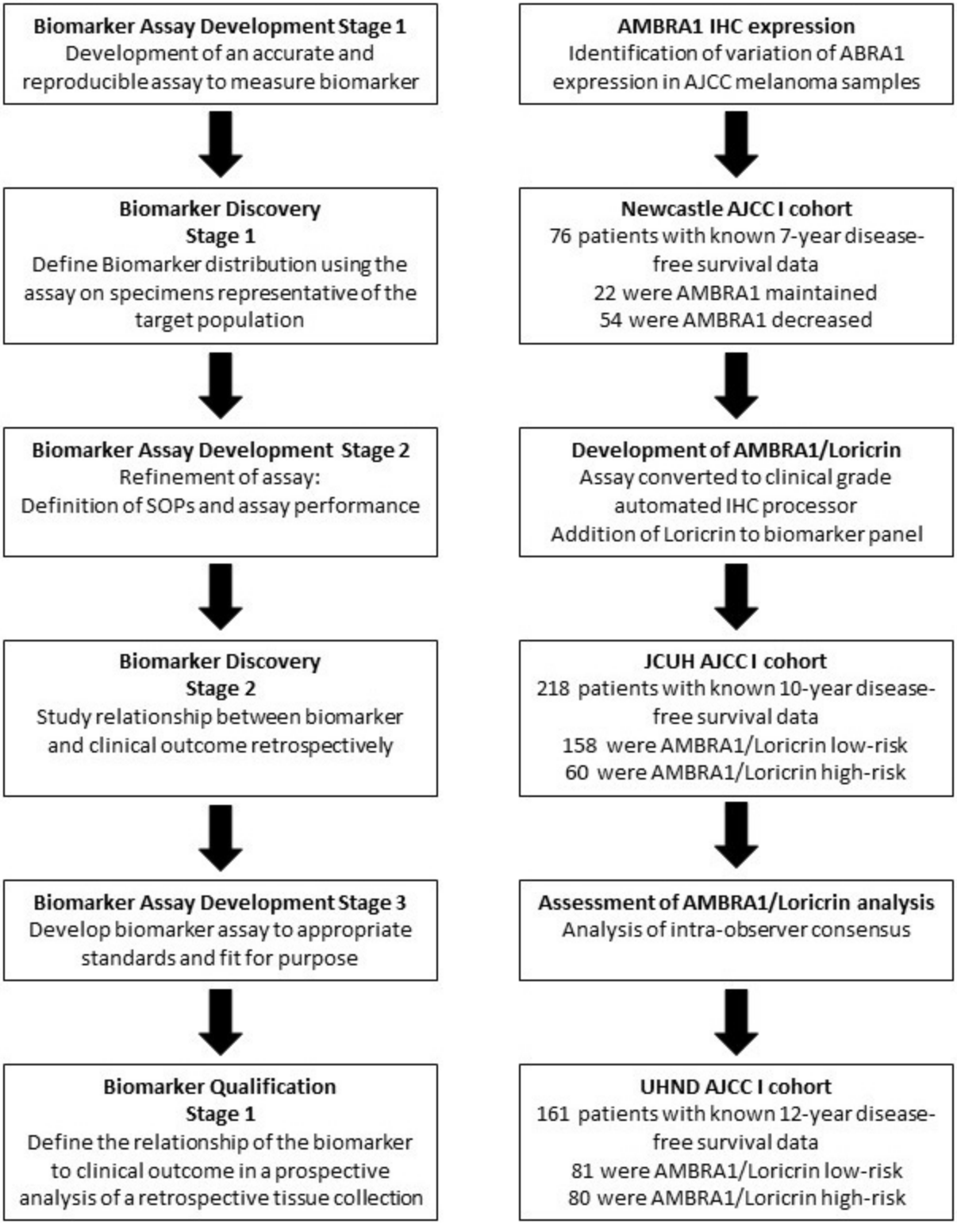
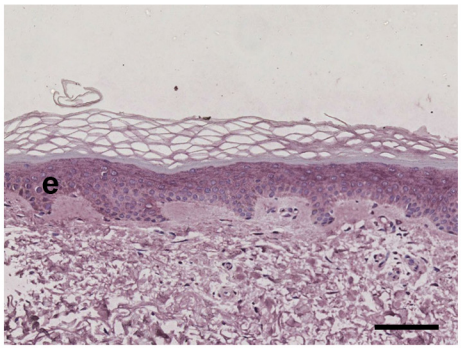

Biomarker Assay Development Stage 1
Development of an accurate and reproducible assay to measure biomarker
AMBRA1 IHC expression
Identification of variation of ABRA1 expression in AJCC melanoma samples
Biomarker Discovery Stage 1
Define Biomarker distribution using the assay on specimens representative of the target population
Newcastle AJCC I cohort
76 patients with known 7-year disease-free survival data
22 were AMBRA1 maintained
54 were AMBRA1 decreased
Biomarker Assay Development Stage 2
Refinement of assay:
Definition of SOPs and assay performance
Development of AMBRA1/Loricrin
Assay converted to clinical grade automated IHC processor
Addition of Loricrin to biomarker panel
Biomarker Discovery Stage 2
Study relationship between biomarker and clinical outcome retrospectively
JCUH AJCC I cohort
218 patients with known 10-year disease-free survival data
158 were AMBRA1/Loricrin low-risk
60 were AMBRA1/Loricrin high-risk
Biomarker Assay Development Stage 3
Develop biomarker assay to appropriate standards and fit for purpose
Assessment of AMBRA1/Loricrin analysis
Analysis of intra-observer consensus
Biomarker Qualification Stage 1
Define the relationship of the biomarker to clinical outcome in a prospective analysis of a retrospective tissue collection
UHND AJCC I cohort
161 patients with known 12-year disease-free survival data
81 were AMBRA1/Loricrin low-risk
80 were AMBRA1/Loricrin high-risk

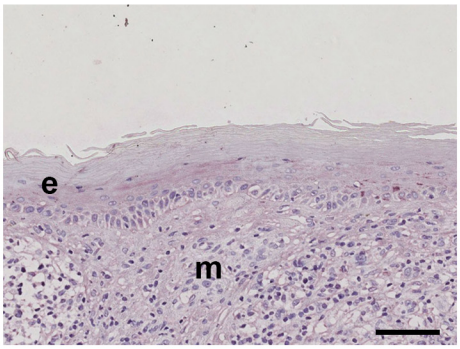
Figure 2

A

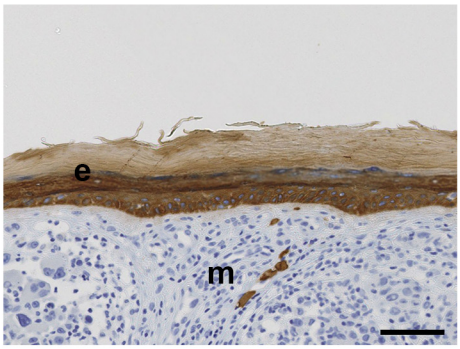
Normal epidermis with maintained epidermal AMBRA1 expression



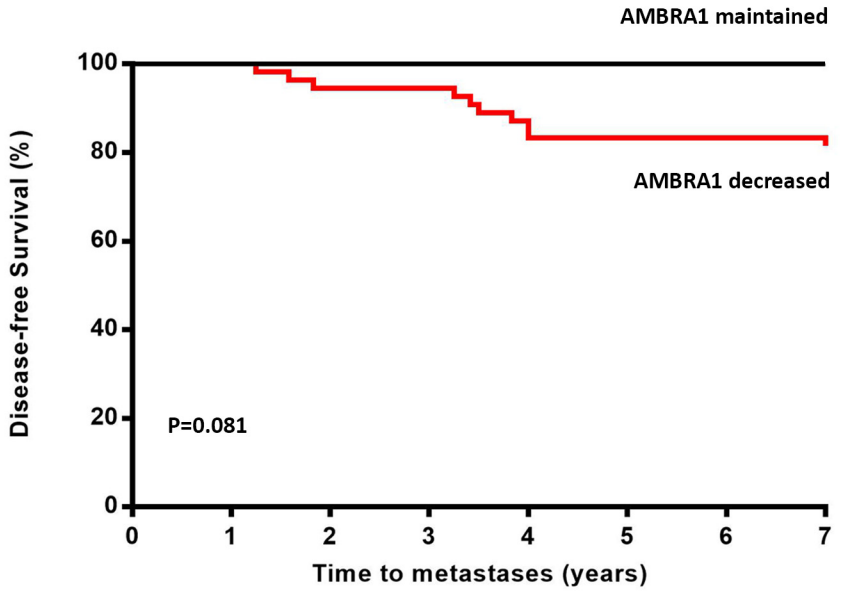

Melanoma with decreased peri-tumoural epidermal AMBRA1 expression




Melanoma with maintained peri-tumoural CK5 expression




B




| No. at Risk | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| AMBRA1 Maintained | 22 | 22 | 22 | 22 | 22 | 22 | 22 | 22 |
| AMBRA1 Decreased | 54 | 52 | 52 | 52 | 47 | 47 | 47 | 45 |

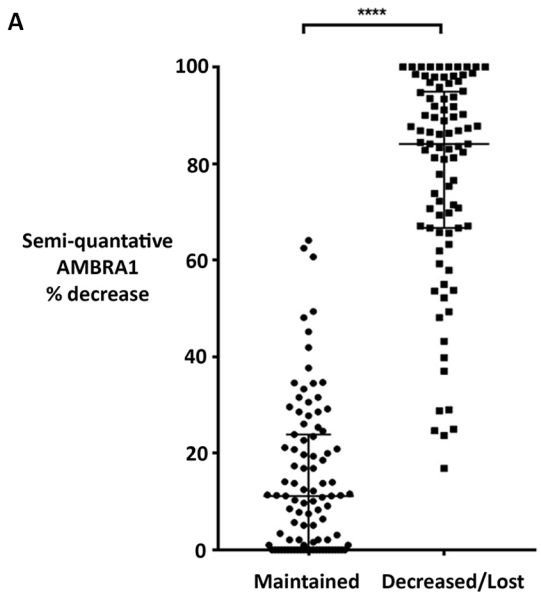
C

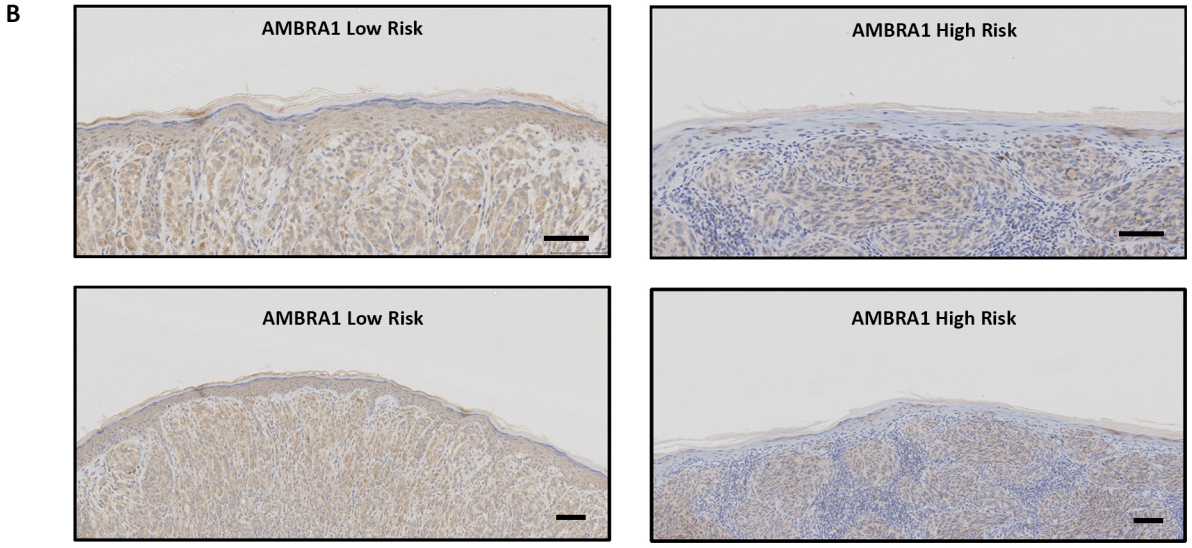
| Variable | Univariate Hazard Ratio (95% CI) | P Value | Multivariate Hazard Ratio (95% CI) | P Value |
|---|---|---|---|---|
| **AMBRA1** | N/A | | N/A | |
| **Breslow Depth** | 7.38 (1.57-34.8) | **0.011** | 5.44 (1.01-29.2) | **0.048** |
| **Age** | 1.03 (0.98-1.08) | 0.25 | 1.01 (0.96-1.07) | 0.61 |
| **Gender** | 1.12 (0.28-4.46) | 0.46 | 1.77 (0.40-7.95) | 0.46 |
| **Mutational Status** | 1.77 (0.57-5.49) | 0.32 | 0.99 (0.28-3.25) | 0.94 |

D

| Sensitivity | Specificity | Positive Predictive Value | Negative Predictive Value |
|---|---|---|---|
| 100.0% | 33.3% | 18.5% | 100.0% |

Figure 3


A
****
100
80
60
40
20
0
Semi-quantative
AMBRA1
% decrease
Maintained
Decreased/Lost


B
AMBRA1 Low Risk
AMBRA1 High Risk
AMBRA1 Low Risk
AMBRA1 High Risk

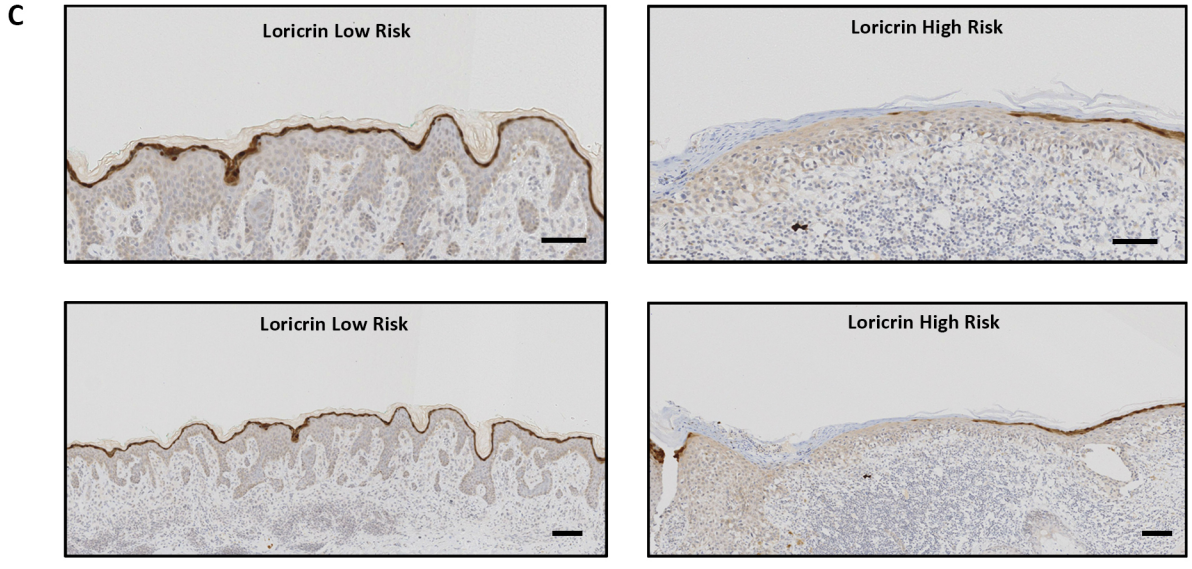
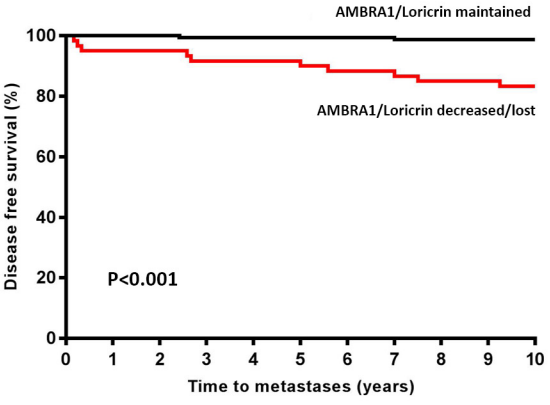

C
Loricrin Low Risk
Loricrin High Risk
Loricrin Low Risk
Loricrin High Risk

**Figure 4**

**A**



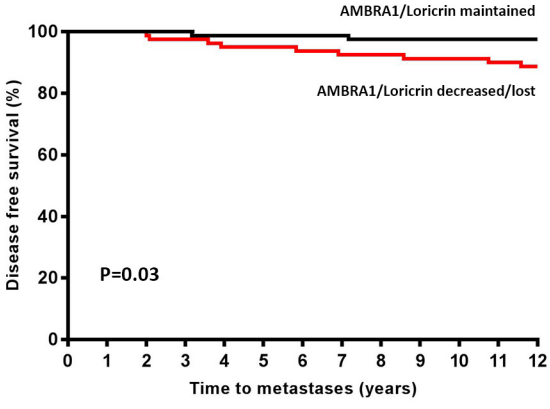

| No. at Risk | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| AMBRA1/Loricrin Low Risk | 158 | 158 | 158 | 157 | 157 | 157 | 157 | 156 | 156 | 156 | 155 |
| AMBRA1/Loricrin High Risk | 60 | 57 | 57 | 55 | 55 | 54 | 53 | 52 | 51 | 51 | 50 |

**B**

| Variable | Univariate Hazard Ratio (95% CI) | P Value | Multivariate Hazard Ratio (95% CI) | P Value |
|---|---|---|---|---|
| **AMBRA1/Loricrin** | 6.53 (2.23-19.1) | **<0.001** | 7.28 (2.36-22.4) | **<0.001** |
| **Breslow Depth** | 3.05 (1.04-8.94) | **0.043** | 1.68 (0.525-5.36) | 0.38 |
| **Age** | 0.992 (0.959-1.03) | 0.66 | 0.974 (0.939-1.01) | 0.15 |
| **Gender** | 1.34 (0.424-4.21) | 0.62 | 1.93 (0.604-6.18) | 0.27 |

| Sensitivity | Specificity | Positive Predictive Value | Negative Predictive Value |
|---|---|---|---|
| 83.3% | 75.7% | 16.7% | 98.7% |

**C**




| No. at Risk | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| AMBRA1/Loricrin Low Risk | 81 | 81 | 81 | 81 | 80 | 80 | 80 | 80 | 79 | 79 | 79 | 79 | 79 |
| AMBRA1/Loricrin High Risk | 80 | 80 | 80 | 79 | 77 | 77 | 76 | 75 | 75 | 74 | 74 | 73 | 71 |

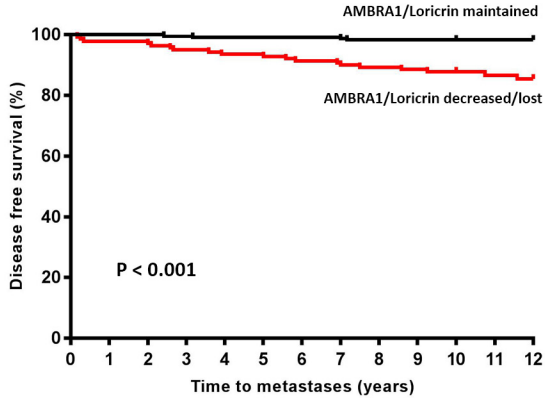
**D**

| Variable | Univariate Hazard Ratio (95% CI) | P Value | Multivariate Hazard Ratio (95% CI) | P Value |
|---|---|---|---|---|
| **AMBRA1/Loricrin** | 2.92 (0.99-8.64) | 0.052 | 2.58 (0.87-7.65) | 0.088 |
| **Breslow Depth** | 13.3 (3.47-51.2) | **<0.001** | 15.6 (3.39-71.8) | **<0.001** |
| **Age** | 0.98 (0.95-1.02) | 0.37 | 0.98 (0.95-1.01) | 0.194 |
| **Gender** | 2.1 (0.64-6.88) | 0.22 | 1.79 (0.55-5.88) | 0.34 |

| Sensitivity | Specificity | Positive Predictive Value | Negative Predictive Value |
|---|---|---|---|
| 81.8% | 52.7% | 11.3% | 97.5% |

# Figure 5

A




| No. at Risk | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| AMBRA1/Loricrin Low Risk | 239 | 239 | 239 | 238 | 237 | 237 | 237 | 236 | 235 | 235 | 234 | 78 | 78 |
| AMBRA1/Loricrin High Risk | 140 | 138 | 137 | 133 | 131 | 130 | 128 | 126 | 125 | 124 | 122 | 72 | 71 |

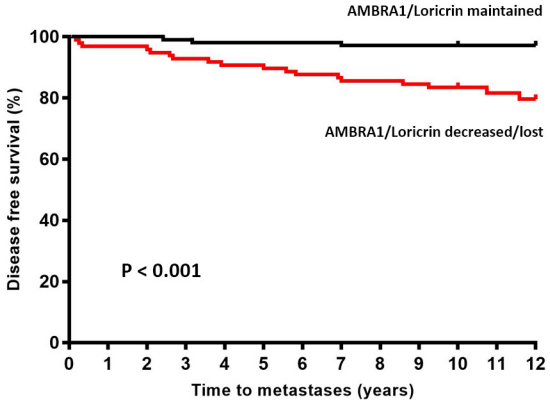
B

## All AJCC I patients

| Variable | Univariate Hazard Ratio (95% CI) | P Value | Multivariate Hazard Ratio (95% CI) | P Value |
|---|---|---|---|---|
| **AMBRA1/Loricrin** | 4.39 (2.05-9.43) | **<0.001** | 3.89 (1.8-8.41) | **<0.001** |
| **Breslow Depth** | 5.63 (2.52-12.6) | **<0.001** | 4.78 (2.01-11.4) | **<0.001** |
| **Age** | 0.98 (0.964-1.01) | 0.35 | 0.978 (0.956-1) | 0.072 |
| **Gender** | 1.66 (0.732-3.76) | 0.23 | 1.69 (0.745-3.85) | 0.21 |

| Sensitivity | Specificity | Positive Predictive Value | Negative Predictive Value |
|---|---|---|---|
| 82.6% | 66% | 13.6% | 98.3% |

C




| No. at Risk | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| AMBRA1/Loricrin Low Risk | 105 | 104 | 104 | 104 | 103 | 103 | 103 | 102 | 102 | 102 | 101 | 25 | 25 |
| AMBRA1/Loricrin High Risk | 97 | 96 | 95 | 92 | 91 | 89 | 87 | 85 | 85 | 84 | 82 | 43 | 41 |

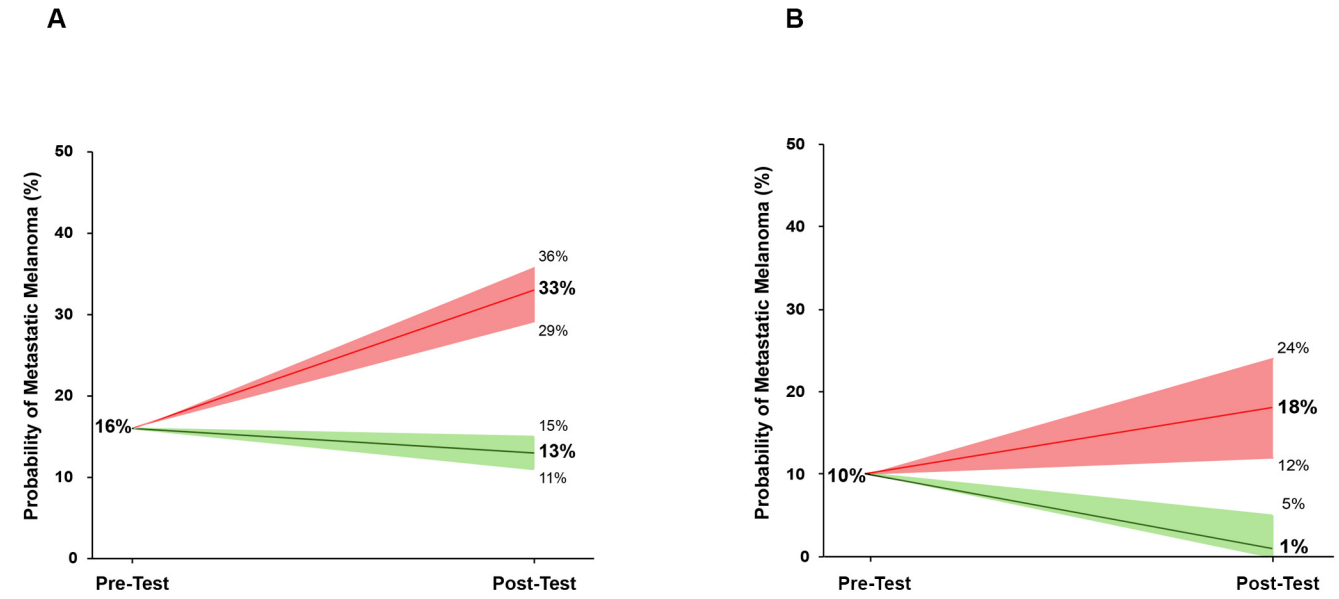
D

## AJCC IB patients only

| Variable | Univariate Hazard Ratio (95% CI) | P Value | Multivariate Hazard Ratio (95% CI) | P Value |
|---|---|---|---|---|
| **AMBRA1/Loricrin** | 3.81 (1.6-9.05) | **0.003** | 4.04 (1.69-9.66) | **0.002** |
| **Breslow Depth** | 3.02 (0.995-9.17) | 0.051 | 2.97 (0.925-9.56) | 0.068 |
| **Age** | 0.98 (0.957-1) | 0.975 | 0.975 (0.952-0.999) | 0.042 |
| **Gender** | 1.23 (0.518-2.92) | 0.64 | 1.32 (0.553-3.14) | 0.53 |

| Sensitivity | Specificity | Positive Predictive Value | Negative Predictive Value |
|---|---|---|---|
| 85.7% | 62.2% | 18.6% | 97.7% |

Figure 6


A
Probability of Metastatic Melanoma (%)
50
40
30
20
10
0
36%
33%
29%
16%
15%
13%
11%
Pre-Test
Post-Test
B
Probability of Metastatic Melanoma (%)
50
40
30
20
10
0
24%
18%
12%
10%
5%
1%
Pre-Test
Post-Test

**Supplementary Methods**

For manual detection of AMBRA1 expression tumours were subjected to pre-optimised IHC analysis (1) with antigen retrieval preformed in 10 mM Tris-Hcl (pH 7.6) and primary antibody binding detected with primary AMBRA1 (Abcam) diluted 1:200, visualised using VIP counterstaining (Vector Labs) .

Antigen retrieval conditions and antibody dilutions for the automated IHC detection of AMBRA1 (Abcam), Loricrin (Abcam) and Cytokeratin 5 (Novocastra) were optimised using a Ventana Benchmark XT automated IHC staining instrument (Ventana Medical Systems Inc.) with antibody binding visualised either with an Optiview DAB Detection Kit (Ventana Medical Systems Inc.) or an ultraView Universal DAB Detection Kit (Ventana Medical Systems Inc.), according to the manufacturers specifications.

Following visual validation of consistent AMBRA1 and Loricrin expression these antibodies were further analysed in the JCUH and UHND tissue cohort samples in the Pathology department of JCUH at concentrations of 1:300 AMBRA1, 1:1500 Loricrin, 1:100 Cytokeratin 5 with final counterstaining in haematoxylin for 8 minutes at room temperature. All IHC stained sections were digitally imaged using automated scanning of slides on a digital slide scanner (Leica SCN400) for subsequent visual and semi-quantitative analysis.

For utility analysis of each biomarker cohort the classification functions for sensitivity, specificity, positive predictive value and negative predictive value were undertaken:

$$\text{Sensitivity} = \frac{\text{Number of true positive}}{\text{Number of true positives + number false negatives}}$$

$$\text{Specificity} = \frac{\text{Number of true negatives}}{\text{Total number of well individuals in population}}$$

$$\text{Positive Predictive Value} = \frac{\text{Number of true positives}}{\text{Number of true positives + number of false positives}}$$

$$\text{Negative Predictive Value} = \frac{\text{Number of false positives}}{\text{Number of true negatives + number of false positives}}$$

For analysis of SLNB outcome data (Figure 6), analysis steps undertaken were:

LRp <- sensitivity/(1 - specificity)  Diagnostic likelihood ratio of a positive test
LRn <- (1 -sensitivity)/(specificity)  DLR n

PreTestOddsP <- prevalence/(1 - prevalence)  Pre test odds of positive result
PreTestOddsN <- (prevalence)/(1 -prevalence)  Pre test odds of negative result

PostTestOddsP <- PreTestOddsP*LRp  Post test odds of positive result PostTestOddsN <- PreTestOddsN*LRn Post test odds of negative result

PostTestProbP <- PostTestOddsP/(PostTestOddsP + 1) – post test probability of positive result = PPV
PostTestProbN <- PostTestOddsN/(PostTestOddsN + 1)  post test probability of negative result = 1-NPV

**Supplementary Figure 1. Loss of AMBRA1 results in deregulated epidermal differentiation.**

(A) Representative Western blot (n=3) for AMBRA1, loricrin, cytokeratin 14 (CK-14) and β-actin protein expression in primary keratinocytes following switch from culture in 0.06mM calcium chloride to culture in high calcium (1.3mM) for 5 days. (B) Cell proliferation (Sulphorhodamine B assay) or representative Western blot (n = 2) for AMBRA1 or β-actin protein expression in CCD-1106 keratinocytes following transfection with AMBRA1 siRNA (si-AMBRA1) or a non-targeting siRNA (si-Ctrl) by reverse transfection and culture for 7 days (for cell proliferation) or for 6 hours and subsequent culture for 5 days (for Western blot). (C) Western blot (n = 3) and RT-qPCR mRNA (n = 4) expression analysis of AMBRA1, Loricrin and GAPDH or RPL13A in primary keratinocytes transfected with control (si-Ctrl) or AMBRA1 (siAMBRA1) siRNA and incubated in high calcium (1.3 mM) for 5 days. Protein levels were quantified by densitometry, normalised to GAPDH, and presented relative to siCtrl (mean ± SD). mRNA expression levels were normalised to RPL13A and presented relative to siCtrl (mean ± SD). Unpaired one-sample T-test; *** $P < 0.001$, ** $P < 0.01$, * $P< 0.05$. (D) Representative IHC images of epidermal AMBRA1 and Loricrin expression in normal skin (Scale bar = 100μm).

**Supplementary Figure 2. Patient demographics and selection pathways**

(A) Demographic data of the Newcastle University Teaching Hospital, James Cook University Hospital and University Hospital of North Durham AJCC Stage I cohorts. (B) Sample selection pathway for the James Cook University Hospital and University Hospital of North Durham cohorts.

**Supplementary Figure 3. Epidermal expression of AMBRA1 and Loricrin in normal skin and overlying benign nevi.**

Representative photomicrographs of immunohistochemical negative control (A), AMBRA1 (B,D) or Loricrin staining (C,D) in the normal epidermis (A,B,C) as well as in the epidermis overlying benign melanocytic naevi (D, E). Scale bars = 100μm.

**Supplementary Figure 4. Scoring system for epidermal AMBRA1 expression.**

Schematic and representative IHC images of AMBRA1 expression peritumoural epidermis compared to matched normal epidermis at the section margins, showing maintained AMBRA1 (A; low risk, score = 0), decreased AMBRA1 (B; high risk, score = 1) and loss of AMBRA1 (C; high risk, score = 2) (m = melanoma, p = peri-tumoural epidermis, n = normal epidermis, scale bars = 100μm).

**Supplementary Figure 5. Scoring system for epidermal Loricrin expression.**

Schematic and representative IHC images of Loricrin expression peritumoural epidermis compared to matched normal epidermis at the section margins, showing maintained Loricrin (A; low risk, score = 0) and complete loss of Loricrin (B; high risk, score = 1) (m = melanoma, p = peri-tumoural epidermis, n = normal epidermis, scale bars = 100μm).

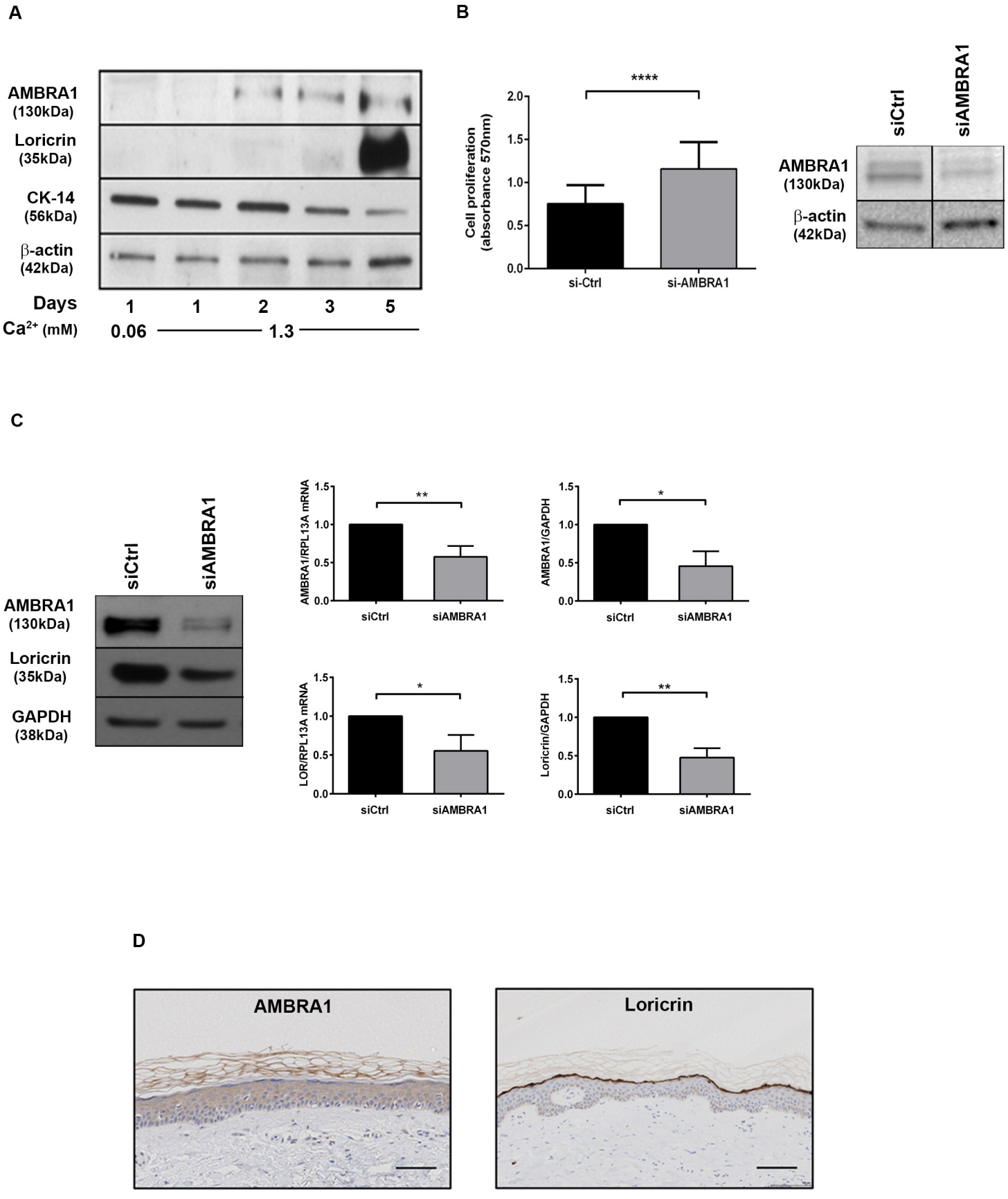
**Supplementary Figure 6. Relationship between Loricrin, and AMBRA1 and Loricrin expression in the Newcastle Discovery Cohort.**

AMBRA1 and Loricrin levels in the peritumoural epidermis of AJCC stage I melanomas were determined by pathologist visual inspection and defined as maintained or decreased. 80-month disease free survival rates were determined with the Kaplan-Meier method and compared by two-sided Log-Rank test for Loricrin analysis only (A; 20% vs 88.9%; P=0.026) or combined AMBRA1/Loricrin (B; 0% vs 100%; P<0.001).

**References**

1. Ellis RA, Horswell S, Ness T, Lumsdon J, Tooze SA, Kirkham N, et al. Prognostic impact of p62 expression in cutaneous malignant melanoma. J Invest Dermatol. 2014;134(5):1476-8.

**Supplementary Figure 1**



**Supplementary Figure 2**

**A**

| Newcastle University Teaching Hospitals Cohort | |
|---|---|
| Cohort size | 76 |
| Male:Female | 36 vs 40 |
| Mean Age (Range) years | 55 (17-82) |
| Mean Breslow Depth (Range) mm | 1 (0.3-2) |
| Mean Time to Metastasis (Range) Months | 40 (15-84) |
| Mutational Status | 26 WT vs 37 BRAF/NRAS mutants |

| James Cook University Hospital | |
|---|---|
| Cohort size | 218 |
| Male:Female | 76 vs 142 |
| Mean Age (Range) years | 55 (13-94) |
| Mean Breslow Depth (Range) mm | 0.92 (0.1-2) |
| Mean Time to Metastasis (Range) Months | 50 (2-111) |

| University Hospital North Durham | |
|---|---|
| Cohort size | 161 |
| Male:Female | 61 vs 100 |
| Mean Age (Range) years | 52 (15-90) |
| Mean Breslow Depth (Range) mm | 0.85 (0.2-2) |
| Mean Time to Metastasis (Range) Months | 68 (24-129) |

**B**

**Sample selection pathway JCUH cohort**

460 AJCC I (2009) samples identified from prior to 2006. 20 patients eventually developed metastases

↓

205 samples excluded as initial histology report unavailable/incomplete or no available follow up data

↓

255 samples with 12 metastases

↓

27 FFPE blocks not available due to damage

↓

228 Samples stained for AMBRA1/Loricrin

↓

8 samples with no visible tumour or ulcerated overlying epidermis

↓

**218 patients included in final analysis with 12 metastases**

**Sample selection pathway UHND cohort**

195 AJCC I (2009) samples identified consecutively from 1st Jan 2003. 16 patients eventually developed metastases

↓

27 blocks unavailable due to damage

↓

168 samples with 13 metastases stained for AMBRA1/Loricrin

↓

7 samples with no visible tumour or overlying epidermal ulceration

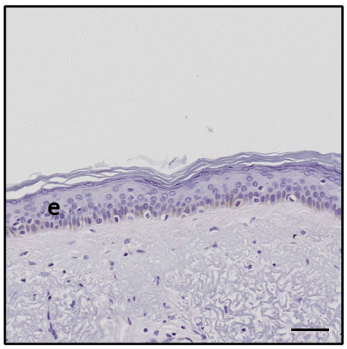
↓

**161 patients included in final analysis with 11 metastases**

**Supplementary Figure 3**

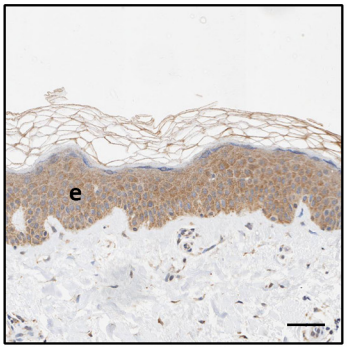
A

Negative control



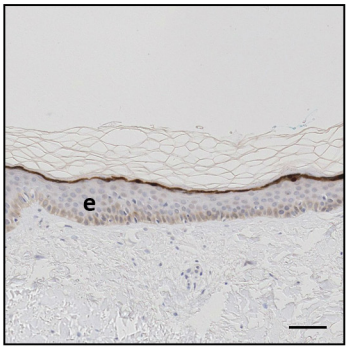

B

AMBRA1 normal epidermis



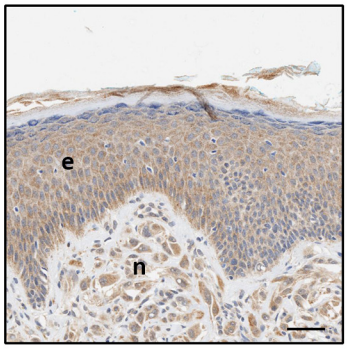

C

Loricrin normal epidermis



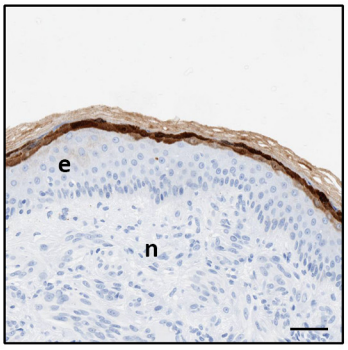

D

AMBRA1 overlying benign naevus




E

Loricrin overlying benign naevus



**Supplementary Figure 4**

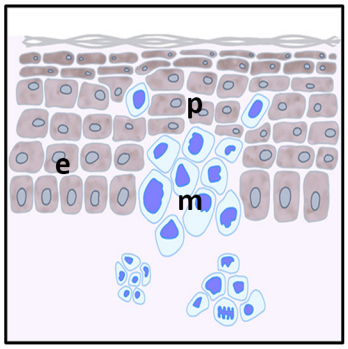
**A**

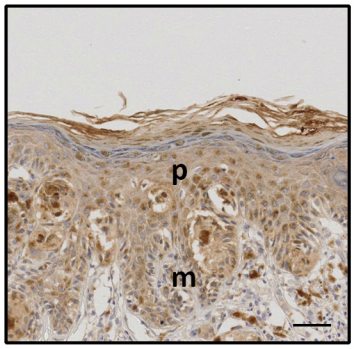
Schematic AMBRA1 expression **maintained** in peri-tumoural epidermis (score = 0)

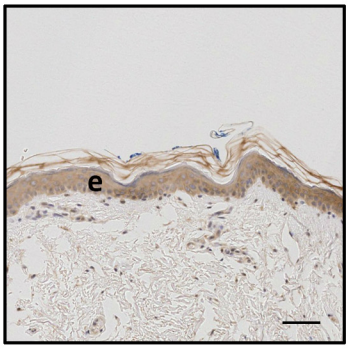
IHC AMBRA1 expression **maintained** in peri-tumoural epidermis (score = 0)

Internal control of AMBRA1 expressionin normal epidermis lateral to melanoma

**Low Risk**




---

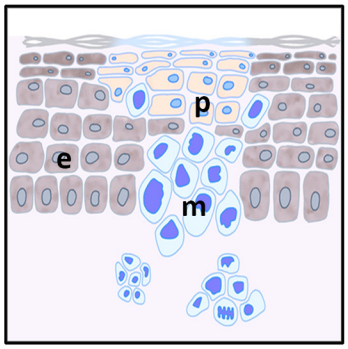
**B**

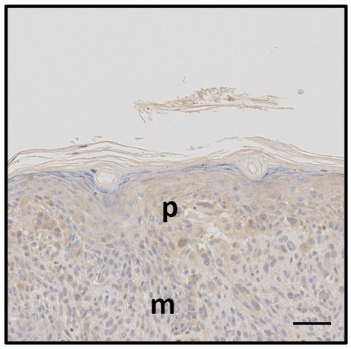
Schematic AMBRA1 expression **decreased** in peri-tumoural epidermis (score = 1)

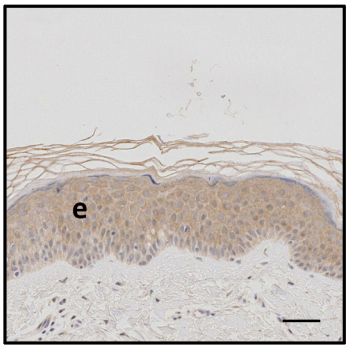
IHC AMBRA1 expression **decreased** in peri-tumoural epidermis (score = 1)

Internal control of AMBRA1 expressionin normal epidermis lateral to melanoma

**HIgh Risk**



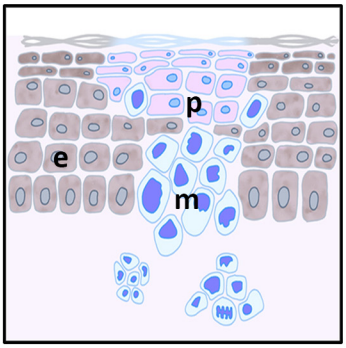

**C**

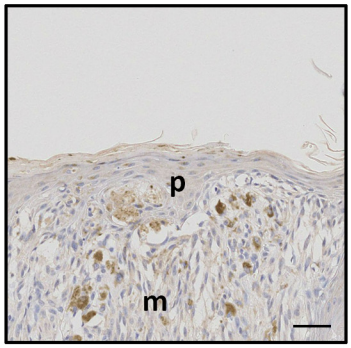
Schematic AMBRA1 expression **lost** in peri-tumoural epidermis (score = 2)

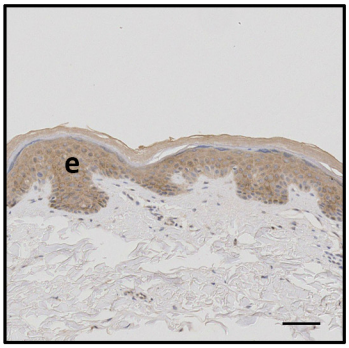
IHC AMBRA1 expression **lost** in peri-tumoural epidermis (score = 2)

Internal control of AMBRA1 expressionin normal epidermis lateral to melanoma

**HIgh Risk**



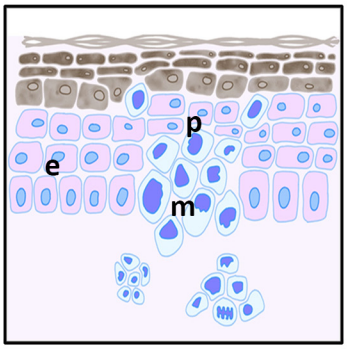



**Supplementary Figure 5**

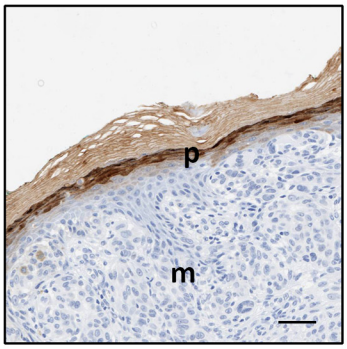
**A**

Low Risk

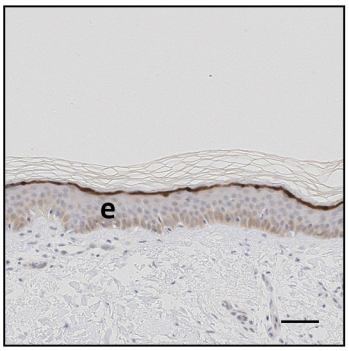
Schematic Loricrin expression **maintained** in peri-tumoural epidermis (score = 0)




IHC Loricrin expression **maintained** in peri-tumoural epidermis (score = 0)




Internal control of Loricrin expressionin normal epidermis lateral to melanoma

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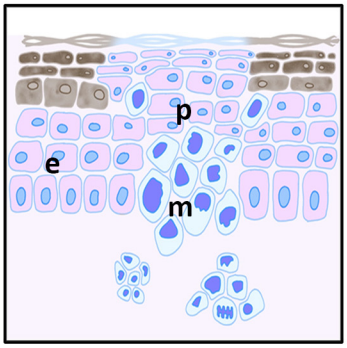
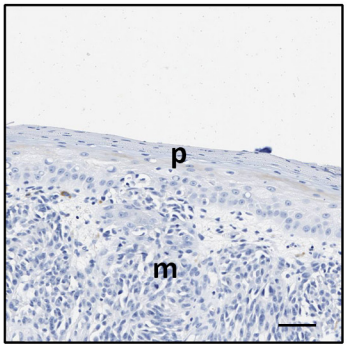
**B**

High Risk

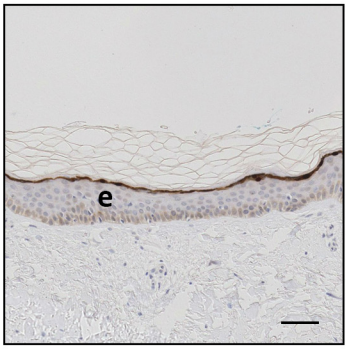
Schematic Loricrin expression **lost** in peri-tumoural epidermis (score = 1)




IHC Loricrin expression **lost** in peri-tumoural epidermis (score = 1)

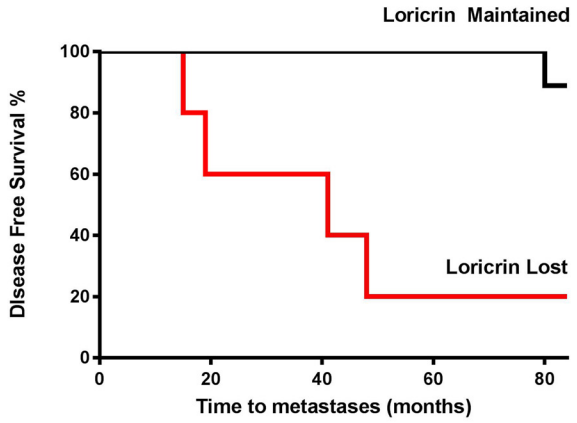



Internal control of Loricrin expressionin normal epidermis lateral to melanoma



**Supplementary Figure 6**

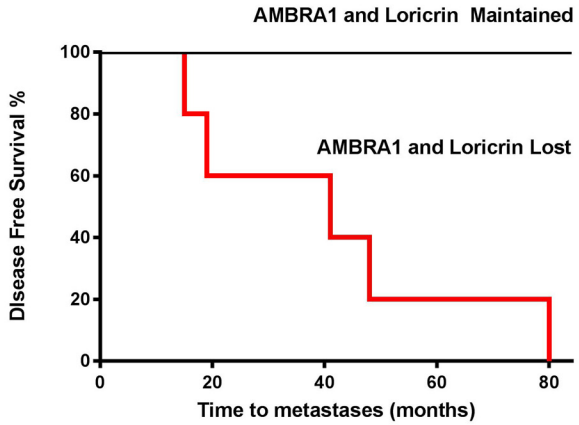
A




| No. at Risk | | | | | |
|---|---|---|---|---|---|
| Loricrin Maintained | 9 | 9 | 9 | 9 | 8 |
| Loricrin Lost | 5 | 3 | 2 | 1 | 1 |

| Sensitivity | Specificity | Positive Predictive Value | Negative Predictive Value |
|---|---|---|---|
| 80.0% | 88.9% | 80.0% | 88.9% |

B




| No. at Risk | | | | | |
|---|---|---|---|---|---|
| A&L Maintained | 8 | 8 | 8 | 8 | 8 |
| A&L Lost | 5 | 3 | 2 | 1 | 0 |

| Sensitivity | Specificity | Positive Predictive Value | Negative Predictive Value |
|---|---|---|---|
| 100.0% | 100.0% | 100.0% | 100.0% |