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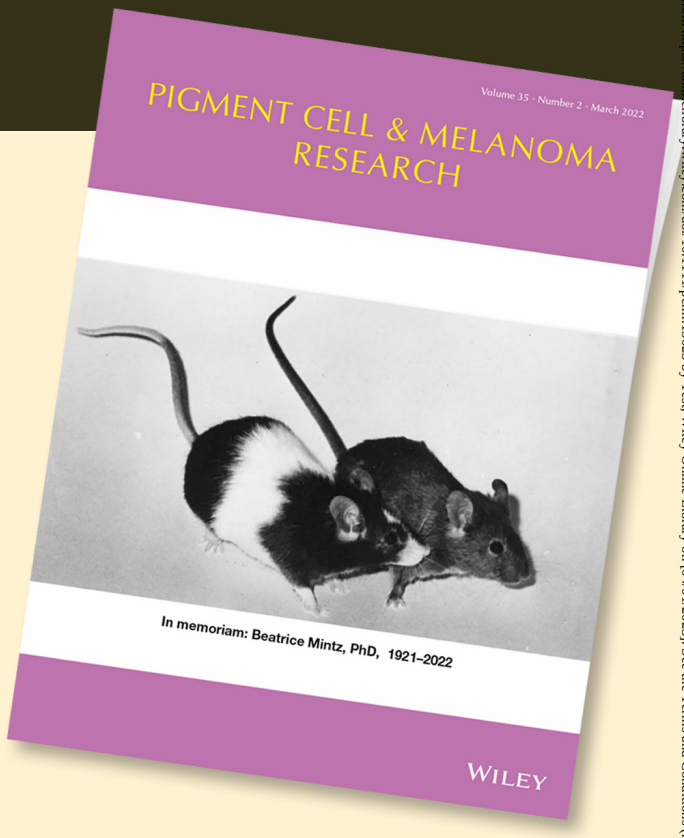

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Ulcerated melanoma: Systems biology evidence of inflammatory imbalance towards pro-tumourigenicity

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Abstract

Microscopic ulceration is an independent predictor of melanoma death. Here, we used systems biology to query the role of host and tumour-specific processes in defining the phenotype. Albumin level as a measure of systemic inflammation was predictive of fewer tumour-infiltrating lymphocytes and poorer survival in the Leeds Melanoma Cohort. Ulcerated melanomas were thicker and more mitotically active (with corresponding transcriptomic upregulated cell cycle pathways). Sequencing identified tumoural *p53* and *APC* mutations, and *TUBB2B* amplification as associated with the phenotype. Ulcerated tumours had perturbed expression of cytokine genes, consistent with protumourigenic inflammation and histological and transcriptomic evidence for reduced adaptive immune cell infiltration. Pathway/network analysis of multiomic data using neural networks highlighted a role for the β-catenin pathway in the ulceration, linking genomic changes in the tumour to immunosuppression and cell proliferation. In summary, the data suggest that ulceration is in part associated with genomic changes but that host factors also predict melanoma death with evidence of reduced immune responses to the tumour.

KEYWORDS
circulating inflammatory markers, copy number, transcriptomics, vitamin D receptor

John Davies and Sathya Muralidhar contributed equally.

1 | INTRODUCTION

Cutaneous melanoma is still increasing in incidence and mortality in many countries. Although immunotherapy has improved outcomes for patients with advanced disease, 40% do not benefit, and the need to better understand the biology of the tumour and host variation in response remains crucial. Microscopic ulceration of primary melanoma is a strong independent predictor of melanoma death, but its biology remains unclear. We have reported evidence previously that ulceration may at least in part, be driven by host systemic inflammation in that obesity, diabetes, vitamin D deficiency and smoking were associated with ulceration, in the Leeds Melanoma Cohort (LMC) (Newton-Bishop et al., 2015). This hypothesis was subsequently supported by an Australian study in which ulceration in thick tumours was associated with diabetes, and that statin use (which is reported to reduce IL-6 levels (Sepehri et al., 2016)) was protective for ulceration (von Schuckmann et al., 2017). IL-6 and other molecules resulting from IL-1β signalling mediate systemic inflammation associated with an increased risk of cardiovascular disease, and suppression of this pathway has been reported to reduce lung cancer deaths (Ridker et al., 2011). Drugging systemic inflammation therefore remains of interest as a potential adjuvant therapy for melanoma.

Genomic studies of primary melanomas are relatively few as a result of the tiny volume of many tumours (Mar et al., 2014) with little evidence of a genomic basis for ulceration (Arbiser, 2014; Mascaro et al., 1984). In a recent study by Koelblinger et al. (2019), ulcerated melanomas had a significantly higher proportion of tumour cells expressing the immunomodulatory protein PD-L1 (programmed death ligand 1), suggesting that the biological processes driving ulceration could impact immune responses. The study also reported increased expression of CD11c (a marker of dendritic cells) and $CD68^+$ and $CD163^+$ macrophages (Koelblinger et al., 2019) in ulcerated tumours. The hypothesis of our study was that tumoural genomic variation plays a significant role in driving the ulceration phenotype in primary melanomas, modified by potentially modifiable environmental exposures. A systems biology approach to its exploration was taken as a model of approaches to understanding complex interactions between host and tumoural variation.

Significance

The role of the tumour microenvironment in progression is increasingly clear. Microscopic ulceration is an ill understood phenotype having prognostic and putatively predictive significance. This paper describes a systems biology investigation of host and multiomic tumour variables associated with the phenotype which identified potentially actionable host factors playing a role in ulceration and highlights the significance of β-catenin signalling in aggressive melanoma.

2 | METHODS

2.1 | Human data and samples

The LMC is a prospective cohort of 2184 primary melanoma patients recruited from a geographically defined area of the UK in the period 2000–2012, see Supplementary Information. The Vitamin D and Immunity Study (VDI) REC reference 13/YH/0237 is a second cohort of 393 primary melanoma patients recruited at diagnosis, making their first visit to the Leeds Melanoma Multidisciplinary Clinic. Here, we report the analysis of ulceration status in association with clinicopathological variables and serum vitamin D levels at diagnosis. The VDI histopathological data were extracted from the summary of the Leeds multidisciplinary team review, which was carried out for all participants according to protocol.

2.2 | Statistical and bioinformatic analyses

See Supplementary Methods.

2.3 | Association of tumour and host variables with ulceration and melanoma-specific survival

The analysis of clinicopathological factors used data collected from the entire LMC cohort of 2184 people and that from 393 participants in the VDI study. Tumour analyses were carried out on the subset of LMC 703 tumours which were large enough to sample yet leave sufficient tumour in the formalin-fixed tumour blocks for subsequent clinical testing if needed. The quantity of DNA and RNA extracted from tumour tissue was limited by tumour size, and therefore, transcriptomic and sequencing data did not overlap completely (Figure S1).

2.4 | Measurement of circulating inflammatory markers and serum vitamin D from blood samples taken at recruitment to the study

Sera stored at −80°C since recruitment (duration of storage 6–18 years) were used to measure C-reactive protein (CRP) and albumin in LMC participants whose tumour genomic data were available (performed at the University of Glasgow) (Salim et al., 2016). This approach was chosen as these measures are established to be stable in cryopreserved serum over time. We computed the Glasgow Prognostic Score (GPS) (Ohmura et al., 2017) derived from CRP/albumin levels, as this is reported to better reflect systemic inflammation and to predict poorer outcomes from colorectal cancer (Nozoe et al., 2014). The individual values and the score were tested as associated with ulceration status and melanoma-specific survival (MSS). The vast majority (93%) were scored 0 (i.e. CRP ≤10 mg/L and albumin ≥35 g/L) and a minority

scored 1 (i.e. CRP >10 mg/L or albumin <35 g/L). Patients who had both a serum elevation of CRP and hypoalbuminaemia were allocated a GPS of 2. Vitamin D levels were measured as reported previously (Newton-Bishop et al., 2009).

In the VDI study, serum vitamin D levels were measured at diagnosis as reported for the LMC (Newton-Bishop et al., 2009) and high sensitivity CRP was measured by the Leeds NHS laboratory in fresh samples.

2.5 | Tumour genomics and ulceration in the LMC

2.5.1 | Genomic data generation

Formalin-fixed paraffin-embedded (FFPE) primary melanomas were sampled using a 0.6 mm diameter tissue micro array (TMA) needle inserted horizontally consistently through the least inflamed part of the invasive tumour. DNA/RNA were extracted from 820 tumour cores (703 unique patients and 117 duplicates) as described previously using Qiagen AllPrep DNA/RNA FFPE kits (Jewell et al., 2015; Nsengimana et al., 2018). Gene expression was quantified using the Illumina DASL Human HT12 v4 array). DNA samples were used to generate copy number data from 303 of the samples as reported (Filia et al., 2019). Tumour DNA was also processed at the Wellcome Sanger Institute to generate somatic variant calls (mutations) from 524 of the primaries compared with matched blood DNA (465 cases) or a high depth control DNA sample filtered using data from ExAc (http://exac.broadinstitute.org/). Samples were selected only on the basis of there being sufficient DNA available. Targeted capture was performed on 554 genes using Agilent SureSelectXT probes as described by Chen et al. (Birkealv et al., under review, *Nature Communications*, December 2021). The baits included 164 melanoma-associated genes, 245 known to be associated with other solid cancers, common melanoma promoter mutations, and 101 genes from the interferon signalling pathway (Gao et al., 2016) (Supplementary Excel File S1). Human leucoyte antigen (HLA) regions were also screened. Sequencing designed to generate copy number data was carried out using the Illumina HiSeq4000 platform, using 75 bp paired-end reads and data were mapped to GRCh37d5 with BWA-MEM 0.7.15 and somatic variants were called using the Caveman algorithm (v.1.11.2). The mutation load was analysed for its association with ulceration, defined as summated non-synonymous mutations. As the melanoma primary samples are small, there was insufficient material to process all samples using the three different platforms and the overlaps are illustrated in Figure S1.

2.5.2 | Analysis of genomic data

Associations between individual mutations detected and ulceration status were tested using logistic regression (univariate and adjusted for thickness, age and sex). A candidate gene approach was also taken to the analysis of expression of genes coding for cytokines and their association with ulceration status. Differential gene expression by ulceration status was used to identify biological pathways associated with ulceration in MetaCore™ (Table 2). The transcriptomic data were also subjected to a bioinformatic inference of the presence of specific immune cell subgroups in tissues (Angelova et al., 2015), in association with ulceration status. Gene level data associated with ulceration status were generated from segmented copy number using GISTIC (Software.broadinstitute.org). Finally then, the copy number and transcriptomic data were combined in a neural network analysis (Abdel-Fatah et al., 2016). The resultant analysis identified 'influencer' genes and 'influenced' genes associated with ulceration, which were then subjected to pathway and network analysis using MetaCore to explore significantly associated biological systems. We did not analyse mutation data in this combined analysis as the samples in common between all three genomic data sets were significantly smaller (Figure S1).

2.5.3 | Utilisation of artificial neural network-based network inference to determine molecular influence and perturbation of molecular interaction networks in merged transcriptomic and copy number data

Here, we implemented a machine learning approach based on an artificial neural network (ANN) combined with a concordance analysis conducted across multiple Monte Carlo data splits (where four levels of cross validation are conducted). We previously showed this to be highly effective at eliminating false discovery and overfitting, while maximising generalisation of the biomarkers identified (Abdel-Fatah et al., 2016) (Figure S2). The concordant set of markers determined was fed into an ANN-based network inference algorithm (ANNi) (Tong et al., 2014). This approach uses each transcript or gene identified with a copy number change, from the enriched set as a network output and all remaining features as network inputs. This approach was repeated for each data point, creating a group of interaction models. The trained, optimised interaction models were then collectively analysed to define an interaction map for the genomic data points (Figure S4), using the summed weights leading from a given input to a given output. The visualisation of interactome network maps of concordant genes was undertaken in Cytoscape (version 2.8). The Pearson correlation coefficient r with a cut-off value of 0.7 was implemented in the algorithm for removing the least significant interaction scores.

3 | RESULTS

3.1 | Clinicopathological data analyses

There were 187 ulcerated tumours in the LMC (of 576 total examined, 32%) and 153 ulcerated in the VDI study (out of 391 total 39%). Ulceration was more common with increasing age in both the LMC and VDI data sets, and this was independent of other variables in the

TABLE 1 Association of clinicopathological factors with ulceration in LMC and VDI data sets

	Leeds Melanoma Cohort (LMC)					Vitamin D and immunity study (VDI)				
	Adjusted for age and sex			Multivariable ($n = 1007$)		Adjusted for age and sex			Multivariable ($n = 368$)	
	N	OR (95% CI)	*p*	OR (95% CI)	*p*	*N*	OR (95% CI)	*p*	OR (95% CI)	*p*
Age (pa)	2147	1.03 (1.02, 1.04)	**<.001**	1.02 (1.00, 1.04)	**.01**	384	1.02 (1.00, 1.03)	**.03**	1.01 (0.99, 1.02)	.4
Sex	2147					384				
Female	1223	1	-	1	-	187	1	-	1	-
Male	924	1.24 (1.00, 1.5)	**.05**	0.89 (0.60, 1.31)	.6	197	0.99 (0.65, 1.49)	1	0.88 (0.53, 1.44)	.6
TILs	1039					370				
Absent	251	1	-	1	-	52	1	-	1	-
Non-brisk	604	0.82 (0.57, 1.17)	.3	1.07 (0.70, 1.65)	.8	261	1.17 (0.63, 2.17)	.6	1.58 (0.78, 3.18)	.2
Brisk	184	0.55 (0.34, 0.92)	**.02**	1.18 (0.65, 2.14)	.6	57	0.68 (0.30, 1.52)	.3	1.22 (0.49, 3.07)	.7
Breslow, mm	2128					382				
<1.0	605	0.28 (0.17, 0.44)	**<.001**	0.29 (0.11, 0.75)	**.01**	44	0.22 (0.06, 0.79)	**.02**	0.31 (0.09, 1.11)	.07
1–0.99	814	1	-	1	-	101	1	-	1	-
2–3.99	474	3.41 (2.57, 4.51)	**<.001**	3.83 (2.51, 5.86)	**<.001****	131	2.04 (1.14, 3.63)	**.02**	1.75 (0.96, 3.21)	.07
>4	235	8.65 (6.19, 12.1)	**<.001**	6.17 (3.64, 10.5)	**<.001*****	106	5.65 (3.05, 10.5)	**<.001**	4.51 (2.27, 8.94)	**<.001**
Site	2147					376				
Trunk	765	1	-	1	-	138	1	-	1	-
Limbs	210	0.74 (0.56, 0.97)	**.03**	0.73 (0.47, 1.13)	.2	56	1.31 (0.79, 2.15)	.3	1.38 (0.79, 2.41)	.3
Head/neck	959	1.00 (0.68, 1.46)	1	0.76 (0.41, 1.43)	.4	155	1.16 (0.60, 2.24)	.7	0.92 (0.44, 1.91)	.8
Sun-protected sites	213	3.98 (2.84, 5.57)	**<.001**	3.96 (2.27, 6.90)	**<.001***	27	2.36 (1.01, 5.54)	**.05**	2.11 (0.79, 5.62)	.1
Mitoses	1844					376				
<5 mm^{-2}	1331	1	-	1	-	198	1	-	1	-
>5 mm	513	4.9 (3.87, 6.28)	**<.001**	2.69 (1.85, 3.89)	**<.001****	178	3.52 (2.26, 5.48)	**<.001**	2.17 (1.31, 3.59)	**.003**
Univariable analysis										
Log coding mutation count per unit	498	0.84 (0.72, 0.99)	**.04**		Uni-variable analysis****					

Note: Data are presented as univariable and multivariable analyses for each data set, for which logistic regression models were fitted with ulceration as the outcome variable. Missing data, for example where mitotic rates had not been reported by the clinical pathologists, explain the differing numbers in the univariable analyses. The total mutation count was available only for 498 individuals in the LMC data set and the statistical power was considerably reduced if this variable was included in the multivariable analysis (274 individuals); therefore, we report only a univariable analysis for this variable. The stars (as below) show the level of significance, however, for variables adjusted for mutation count. Associations significant at the 5% level or higher are highlighted in bold.

CI, confidence interval; OR, odds ratio.

*Significant at $p < 0.05$ after adjustment for log(total mutation count), $n = 274$.; **Significant at $p < 0.01$ after adjustment for log(total mutation count), $n = 274$.; ***Significant at $p < 0.001$ after adjustment for log(total mutation count), $n = 274$.; ****OR 0.99 (0.74,1.33), $p = 1$ in multivariable model.

larger LMC data set (Table 1). Ulceration was more common in men but this was not independent of other factors. Vitamin D deficiency and smoking were independently associated with ulceration in the LMC as previously reported (Newton-Bishop et al., 2015). Increased body mass index (BMI) was associated with ulceration odds ratio (OR) 1.03 per BMI unit (1.00–1.05), $p = .02$ but, after taking into account vitamin D status, the effect size was less significant (OR 1.02 per unit [0.99–1.04] $p = .2$).

Although the VDI study was very much smaller (having only 18 type II diabetics and 26 current smokers) than the LMC therefore reducing statistical power, the VDI data were consistent with the previous observation of a role for vitamin D. The OR for ulceration in vitamin D deficiency was = 1.18 (0.42, 3.26), $p = .8$ and for MSS: hazard ratio (HR) = 1.48 (0.46, 4.81), $p = .5$, adjusted for age and sex. There was no significant association with sex or smoking and there was a borderline association between BMI and ulceration status in this data set (OR 1.03 [1.00, 1.04], $p = .07$) after adjusting for age and sex.

3.1.1 | Clinicopathological factors and mutation load associated with ulceration

Ulceration was independently more common in thicker tumours and was associated with a higher mitotic rate (in both LMC and VDI studies) (Table 1). Ulceration was more common in tumours arising in sun-protected sites such as acral and genital tumours in the LMC data set independent of thickness and age: OR for ulceration was 3.96 95% confidence interval (CI) 2.27, 6.90, $p < .001$ in the LMC multivariable analysis with support for this in the smaller VDI data set. Brisk tumour-infiltrating lymphocytes (TILs) (as reported in clinical reports) were less common in ulcerated tumours (OR 0.55 95% CI 0.34, 0.92, $p < .02$) in the LMC in a univariable analysis, but this was not significant in the multivariable analysis nor in the VDI study. Total tumour mutation load data were available for 498 participants in whom blood test results were also available, and in a univariable analysis, there was evidence that ulceration was less frequent in tumours with a higher mutation load. This did not, however, persist in the multivariable analysis, which was then a relatively small data set and therefore inadequately powered.

Vascular invasion was seen more frequently in the ulcerated tumours ($p = .0003$) in the LMC data set, and this was independent of tumour thickness $p < .001$. In the smaller VDI data set the difference did not reach statistical significance ($p = .3$) (Table S1). Tumour regression was not consistently commented on in the clinical histopathology reports but was assessed in a single observer analysis of the LMC tumours sampled for genomic studies. Ulcerated tumours were more likely to have regression when the whole slide was considered, compared to non-ulcerated tumours (chi-squared test, $p = .001$) and the depth of regression was also more likely to be greater within ulcerated tumours (whole tumour measure: none, <0.71 mm, 0.71 to <1.14 mm, 1.14 to <1.63 mm, ≥1.63 mm, Fisher's exact test, $p = .00001$).

3.1.2 | Examination of the cored tumour site by single observer

There was no difference in the presence or absence of an immune infiltrate detected histologically in the region of the tumour sampled for genomic studies (Fisher's exact test, $p = .4$) comparing ulcerated with non-ulcerated tumours. Ulcerated tumours were, however, more likely to have a core immune infiltrate classified as 'none or barely perceptible' compared to non-ulcerated tumours (Fisher's exact test, $p = .001$). In terms of specific subtypes of immune cells, ulcerated tumours were less likely to have lymphocytes (Chi-squared test, $p = .0005$) or macrophages (chi-squared test, $p = .0003$) but more likely to contain neutrophils within the core, although the absolute numbers were very small (Fisher's exact test, $p = 3 \times 10^{-6}$). There were only 12 cases with neutrophils in the core and 7 of these were ulcerated, representing 4% ulcerated cases, compared to 0.9% non-ulcerated cases.

3.1.3 | Clinicopathological factors and MSS in the LMC

Table S2 shows the analysis of clinicopathological factors predictive of death from melanoma in the mature LMC and as was expected, age, tumour thickness, tumour site, mitotic rate and TILs were independently predictive. Ulceration was significantly predictive only in the univariable analysis. The data are presented in the table as univariable and multivariable analyses except for mutation load as this factor was measured only in a subset of tumours. In a univariable analysis, a higher mutation load was significantly associated with fewer melanoma deaths. We will report detailed analysis of associations with MSS in the VDI data set when the survival data are more mature.

3.1.4 | Ulceration and death from causes other than melanoma in the LMC

We looked at ulceration and death from causes of death other than melanoma as we had hypothesised that ulceration may also predict deaths related to diseases mediated by systemic inflammation (Table S3). There were 153 non-melanoma deaths in 2018 participants in this data set, and the majority occurred in individuals with diseases associated with systemic inflammation. Of these, 2 had autoimmune disease, 56 cardiovascular events (including cerebrovascular accidents), 10 had chronic lung disease, 11 chronic neurological disease including Alzheimer's, 56 died of other cancers and there were 14 additional deaths caused by rare events such as trauma or the cause of death was unclear. Death from causes other than melanoma in participants with ulcerated tumours was higher in the LMC overall HR 1.47 95% CI (0.98–2.21) $p = .06$ although this was not statistically significant when the analysis adjusted for age and sex HR = 1.14, 95% CI (0.76–1.72), $p = .5$. This was a little stronger in males HR 2.03 95% CI (1.25–3.31), $p = .004$, although this also lost significance when adjusted for age and sex HR = 1.57, 95% CI (0.96–2.57), $p = .1$.

(a) Pvalue for association between variables

Factor	CRP	Albumin	GPS index	Log(CRP/ALB)
Ulceration	0.64	0.37	0.64	0.64
TILs	0.46	0.20	0.16	0.54
AJCC stage	0.69	0.27	0.58	0.35

(b) Association between TILs, CRP and Albumin

	Brisk TILs	Non-Brisk TILs	No TILs	P
Albumin<43.3g/L, n (%)	24 (11.1)	156 (71.9)	37 (17.0)	0.02
Albumin>43.3g/L , n (%)	42 (20.9)	127 (63.2)	32 (15.9)	
CRP<1.5mg/L , n (%)	35 (16.5)	141 (66.5)	36 (17.0)	0.89
CRP>1.5mg/L , n (%)	31 (15.1)	142 (68.9)	33 (16.0)	

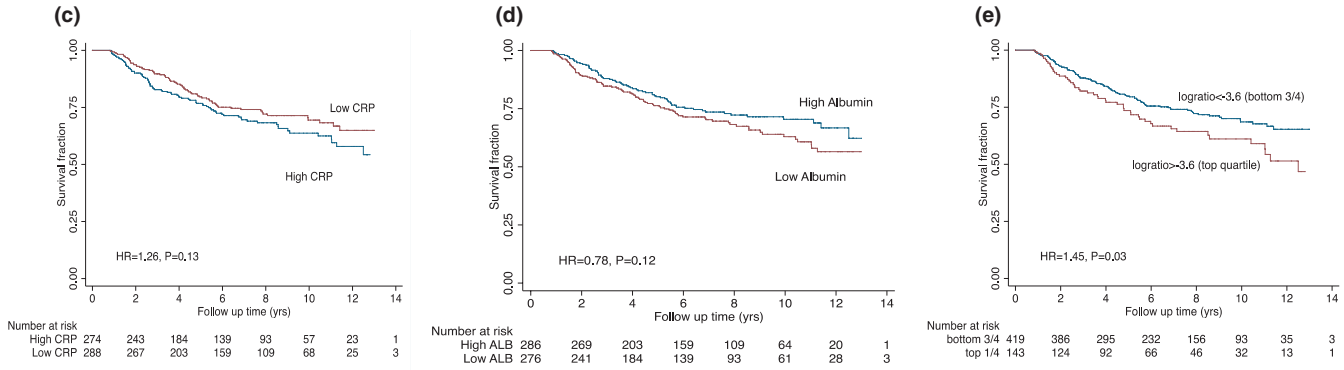


FIGURE 1 Associations between inflammatory markers CRP and albumin in the LMC participants whose tumours were sampled. Analysis of circulating inflammatory markers in 695 participants in the LMC whose tumours were analysed. (a) The *p* values for tests of association between CRP, albumin levels and the GPS and key variables, tumour ulceration, TILs and AJCC stage. GPS = 0 if CRP ≤10 mg/L and albumin ≥35 mg/L. GPS = 1 if either CRP was higher than range or albumin lower and GPS = 2 if both CRP and albumin were higher than threshold. (b) Association between albumin and CRP levels and the presence of brisk TILs in the primary tumours: here, the analysis was stratified at the median. (c) Kaplan–Meier curve for MSS by CRP level. (d) Kaplan–Meier curve for MSS by albumin and (e) Kaplan–Meier curve for MSS by log ratio2 ratio CRP/albumin. CRP, C-reactive protein; GPS, Glasgow Prognostic Score; LMC, Leeds Melanoma Cohort; MSS, melanoma-specific survival; TIL, tumour-infiltrating lymphocyte

3.2 | Circulating inflammatory markers

When analysed on a continuous scale, blood CRP and albumin in 695 samples from the LMC showed no significant association with ulceration, TILs, AJCC stage (Figure 1a) or MSS (Figure 1a–d). However, when dichotomised on the median, 11% of participants whose albumin level was lower than the median, had brisk TILs compared to 21% of those with higher levels ($p = .02$, Figure 1b). Ninety-three percent were scored 0 using the Glasgow Predictive Score (GPS) (i.e. CRP <10 mg/L and albumin >35 g/L), 7% scored 1 (i.e. CRP >10 mg/L or Albumin <35 g/L), and none scored 2. We tested the log2(ratio) of CRP/albumin against MSS, generating a survival curve for the top quartile compared with the others (Figure 1e). All 45 patients with GPS = 1 were in the top quartile of the ratio, with an additional 128 with GPS = 0. Neither the GPS nor the ratio predicted ulceration status: values for the GPS score ($p = .64$), AJCC stage ($p = .58$) or TILs ($p = .16$), but GPS predicted MSS (HR = 1.6, $p = .05$) as did the log2ratio (Figure 1e). Taken individually, CRP and albumin were negatively correlated with each other (Spearman rho = −.27) and had significant opposite and independent associations with MSS: HR = 1.1 ($p = .02$) per CRP unit (log2 scale) and HR = 0.39 ($p = .02$) per albumin unit (log2 scale). These effects were independent of the AJCC stage, although they fell short of significance when dichotomised on the median (Figure 1).

In the VDI study, 372 samples had measurable CRP and 386 samples measurable fibrinogen. No significant associations were seen with ulceration for either CRP (adjusted for age, sex OR = 1.00 95% CI 0.37, 2.66, $p = 1$) or fibrinogen (adjusted for age, sex OR = 1.58 95% CI 0.85, 2.91, $p = .1$). Nor were associations seen with presence of brisk TILs for either CRP (adjusted for age, sex OR = 0.35 95% CI 0.05, 2.74, $p = .3$) or fibrinogen (adjusted for age, sex OR = 1.37 95% CI 0.59, 3.19, $p = .5$). No significant associations were seen for either CRP or fibrinogen with TILs (categorised as absent, non-brisk, brisk).

3.3 | Tumour genomics and ulceration in the LMC

3.3.1 | Mutations and ulceration status

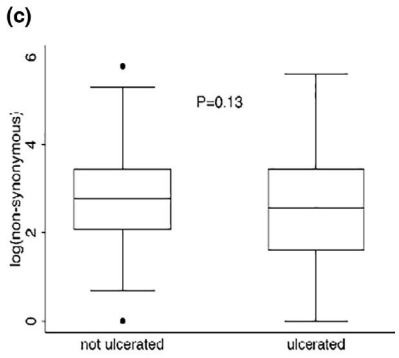
In the LMC data, the commonest mutations detected were *TERT* promoter mutations along with coding mutations in *BRAF*, *CDKN2A* and *NRAS*. These, and all the other coding mutations identified were tested individually for association with ulceration. A number of them showed a marginal association with ulceration in a univariate analysis and after adjusting for tumour thickness, mitotic rate and age of patient at diagnosis, although none remained significant when the data were corrected for multiple testing. Figure 2a shows the mutations

(a)

gene	Tumours mutated	Univariable analysis OR	95%CI_low	95%CI_up	P	Adjusting Breslow thickness, age and sex OR	95%CI_low	95%CI_up	P
APC	21	0.09	0.01	0.69	0.001	0.09	0.01	0.68	0.020
TP53	52	2.52	1.34	4.73	0.004	2.32	1.16	4.62	0.017
ARHGAP35	16	0.12	0.02	0.91	0.006	0.10	0.01	0.82	0.032
BRD7	13	6.32	1.30	30.78	0.011	5.06	0.98	26.15	0.053
CHD3	21	0.21	0.05	0.92	0.013	0.22	0.05	1.03	0.054
ARHGAP21	29	2.77	1.22	6.32	0.014	1.80	0.74	4.38	0.195
NFATC4	25	2.87	1.21	6.77	0.015	2.92	1.148	7.42	0.024
ALK	37	0.34	0.13	0.92	0.019	0.33	0.11	0.94	0.037
ITGA4	32	0.34	0.13	0.92	0.019	0.28	0.10	0.78	0.015
SVEP1	75	0.51	0.28	0.93	0.022	0.49	0.26	0.94	0.032
TCF4	34	0.38	0.15	0.94	0.023	0.32	0.11	0.93	0.035
HDLBP	12	5.38	1.07	26.97	0.025	11.06	1.26	97.30	0.030
PYHIN1	18	0.24	0.05	1.07	0.028	0.24	0.05	1.23	0.086
BAZ2B	23	2.86	1.09	7.52	0.031	2.62	0.89	7.69	0.080
TRPA1	17	3.26	1.07	9.88	0.032	3.37	1.03	10.97	0.044
ATM	19	0.31	0.09	1.09	0.041	0.23	0.06	0.88	0.032
NF1	43	0.45	0.20	1.02	0.042	0.36	0.15	0.87	0.023
DLG2	32	0.41	0.17	1.03	0.043	0.37	0.14	0.99	0.047

(b)

Ulceration status	TP53 No mutation	TP53 1 mutation	TP53 2 mutations	APC No mutation	APC 1 mutation	APC 2 mutations
Ulcerated	141 (34.3%)	21 (52.5%)	4 (100%)	165 (38%)	1 (6%)	0 (0%)
Non-ulcerated	270 (66%)	19 (47.5%)	0 (0%)	271 (62%)	15 (94%)	3 (100%)
P (Fisher)			0.002			0.007



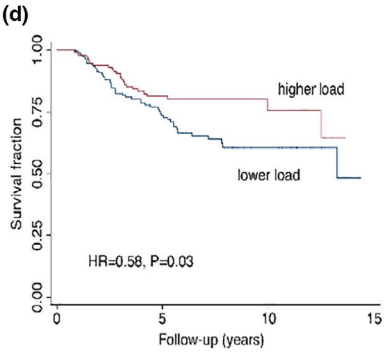


FIGURE 2 Mutations associated with ulceration status. Association between non-synonymous mutations detected in 455 tumours subjected to targeted sequencing, and ulceration or MSS. (a) The most significant associations between individual gene mutations (presence/absence) and ulceration status, adjusted and unadjusted for covariates (not corrected for multiple testing). (b) Heterozygous versus homozygous mutations in the two genes most strongly associated with tumour ulceration in 166 ulcerated and 289 non-ulcerated tumours. (c) Lack of association between ulceration and the load of non-synonymous mutations. (d) Association between the load of non-synonymous mutations and MSS. Comparisons were made between samples with mutation loads recorded at above or below the median. MSS, melanoma-specific survival

significantly associated with ulceration (unadjusted $p < .05$ from at least 10 tumours with one or more mutations). *TP53* and *APC* were the most significantly associated with ulceration with opposing associations: mutations in *TP53* increased the odds of ulceration 2.5-fold (unadjusted $p = .004$) while *APC* mutations decreased the odds of ulceration 10-fold (unadjusted $p = .001$, see Figure 2a,b). These associations remained significant after adjusting for thickness, age and sex. APC gene expression was borderline significantly lower in the tumours with *APC* mutations ($p = .06$) as was also true for *TP53* ($p = .03$). Of the most common driver mutations, those in *BRAF* and *NRAS* mutations were neutral in terms of MSS while *NF1* was protective for death (adjusted OR = 0.36, $p = .02$, see Figure 2a). The total coding mutation load in the 554 genes sequenced was not associated with ulceration status (Figure 2c) but a higher load predicted an improved MSS (Figure 2d). There was no association of mutation load with TILs ($p = .21$) or AJCC stage ($p = .51$).

3.3.2 | Transcriptomic data

We then sought to identify biological pathways associated with ulceration in the 703 LMC primary melanomas for which transcriptomic data were available. A whole-genome univariate test (Mann–Whitney) revealed that 4660 genes varied in expression significantly with ulceration (Benjamini–Hochberg false discovery rate 5%). Genes whose expression was significantly higher in ulcerated tumours ($n = 2681$) were enriched for pathways related to cell proliferation, such as mitotic prometaphase, signalling by Rho-GTPases, cell cycle checkpoints and mitochondrial translation. Conversely, the genes which had significantly lower expression in ulcerated tumours ($n = 1979$) were enriched for pathways related to the tumour microenvironment including immune signalling such as extracellular matrix (ECM) organisation, ECM–receptor interaction, cytokine–cytokine receptor interaction, interferon gamma signalling and cell adhesion pathways such as β1 integrin cell surface interactions and cell adhesion molecules (Table 2). The inferred immune cell subgroups (Table 3) showed evidence for increased signals of eosinophils and activated CD4 cells in ulcerated tumours, but the data suggested that overall signals from immune cell subgroups were largely reduced. The subgroups reduced in ulcerated tumours included natural killer and T cells including Th1 and Th2 cells.

3.3.3 | Copy number data

The analysis of 10K windows did not identify any loci which were significantly associated with ulceration after correction for

TABLE 2 Pathway analysis of the tumour genes differentially expressed in ulcerated compared with non-ulcerated tumours

Pathway associated	Direction of association of genes up- or downregulated in ulcerated tumours	Significance of the association (*p*-value)
Mitotic prometaphase(R)	Upregulated	1.11×10^{-16}
Mitotic metaphase and anaphase(R)	Upregulated	1.11×10^{-16}
Signalling by Rho GTPases(R)	Upregulated	2.22×10^{-16}
Mitotic G1-G1/S phases(R)	Upregulated	1.97×10^{-13}
Cell cycle checkpoints(R)	Upregulated	2.35×10^{-13}
Synthesis of DNA(R)	Upregulated	2.51×10^{-11}
S Phase(R)	Upregulated	4.18×10^{-11}
Mitochondrial translation(R)	Upregulated	2.80×10^{-10}
Cell cycle(K)	Upregulated	3.76×10^{-10}
HDR through Homologous Recombination (HR) or single strand annealing (SSA)(R)	Upregulated	4.00×10^{-10}
Extracellular matrix organisation(R)	Downregulated	1.45×10^{-11}
Pathways in cancer(K)	Downregulated	9.67×10^{-10}
ECM–receptor interaction(K)	Downregulated	1.38×10^{-9}
Cytokine–cytokine receptor interaction(K)	Downregulated	9.87×10^{-9}
Interferon gamma signalling(R)	Downregulated	3.48×10^{-8}
Beta1 integrin cell surface interactions(N)	Downregulated	1.25×10^{-7}
HTLV-I infection(K)	Downregulated	2.54×10^{-7}
Cell adhesion molecules (CAMs)(K)	Downregulated	3.73×10^{-7}
PI3K-Akt signalling pathway(K)	Downregulated	4.57×10^{-7}
Inflammatory bowel disease (IBD)(K)	Downregulated	4.61×10^{-7}

Note: Candidate gene analysis looking at cytokine genes and inferred immune cell subgroups: A candidate gene analysis was carried out looking at the relationship between ulceration and 133 genes encoding cytokines and chemokines which showed that ulcerated tumours had lower expressions levels for the majority of genes tested; 13 genes whose expression was, however, increased in ulcerated tumours included genes coding for IL-1β and IL-6 (Table S5). Inferring immune cell subtypes (Angelova et al., 2015) showed that 15 inferred cell types were reduced in the ulcerated compared with non-ulcerated tumours, these included dendritic cells as well as T cell and NK cell subsets.

multiple testing but the genes associated with ulcerations most significantly are provided in the Supplementary Excel File S2 and summarised in Table S4. The measure of total copy number load (fraction of genome altered by ulceration status), however, showed a higher load in ulcerated tumours (Figure S2), fold change 1.3, $p = .004$. Genes identified as having copy number differences at loci associated with ulceration at the $p < .01$ significance level were analysed using MetaCore. 1787 windows associated with a different gene name were processed and those genes with differential copy number signals in association with ulceration status, identified evidence of pathways associated with cytoskeleton remodelling, cell adhesion and neurophysiological processes as associated with ulceration (Figure S2b). The most significantly identified pathway, Cytoskeleton Remodeling Keratin filaments is illustrated in Figure S2c. The figure shows that copy number changes related to genes coding for tubulin beta, tubulin heterodimers, desmoplakin and plakofilin 1 were associated with ulceration. The strongest signal was from tubulin genes, multiple tubulin genes being identified in the list of 10K windows most associated with ulceration, the most statistically significant being *TUBB2B*. This locus was commonly amplified in the whole data set, being seen in 130 of the 303 tumours, and these were uniformally non-focal (Figure S3). Ulcerated tumours were more frequently amplified, being seen in 52% of 110 ulcerated lesions compared with 38% of the non-ulcerated OR 1.87 (compared with no copy number changed $p = .01$) when the traces were graded visually. A computational measure of segmented data showed that the copy number value fold change in association with ulceration was fold change (FC) 1.14, $p = .0007$. Melanomas arising in non-sun exposed sites (rare melanomas) were especially frequently ulcerated, 18/26 (69%) but there was no significant difference in frequency of genomic amplification with ulceration.

Plakophilin 1 (*PKP1*) was the gene whose expression was second most strongly associated with ulceration. Overall amplification was seen in 83/303 tumours in traces graded visually and in all the amplification was non-focal. There were no statistically significant differences in amplification between ulcerated and non-ulcerated tumours $\chi^2(2) = 1.81$, $p = .48$ but the computational measure of segmented data showed that there was amplification of the locus on average in ulcerated tumours FC = 1.05, $p = .03$. There was borderline negative association between ulceration and amplification of *PKP1* in tumours originating in

TABLE 3 In silico analysis of the expression of genes coding for inferred immune cell subgroups and ulceration status

Immune subtype	Direction of association of inferred cell types up- or downregulated in ulcerated tumours	Significance of the association (*p*-value)
Eosinophil	Upregulated	.00014
Activated_CD4	Upregulated	.00134
$CD56^{dim}$ NK cells	Downregulated	6.70×10^{-7}
Mast_cells	Downregulated	.00023
Th2	Downregulated	.00027
Central_memory_CD4	Downregulated	.0046
Th1	Downregulated	.0047
T follicular helper cells	Downregulated	.0065
$CD56^{bright}$ NK cells	Downregulated	.0082
T_cells	Downregulated	.012
NKT cells	Downregulated	.018
Activated_B_cells	Downregulated	.018
Gamma-delta T cells	Downregulated	.024
Dendritic cells	Downregulated	.041
Myeloid-derived suppressor cells	Downregulated	0.048

Note: Bioinformatic approach to inferring immune cell tumour infiltration was performed as described by Angelova et al. (2015). The scores for 15 immune cell subgroups, derived from significantly differentially genes held to be specific to those cells, are shown where there was a significant difference in association with ulcerated tumours at a *p*-value <.05, without correction for multiple testing. The estimated scores for ulcerated tumours for all but two immune cell types were significantly reduced in this analysis compared with the non-ulcerated tumours.

non-sun exposed tissues, where the traces were examined visually OR = 0.2, $p = .08$ (test for trend of odds). Examples of the tracings are shown in Figure S3.

3.3.4 | The neural network analysis of transcriptomic data and genes at loci for which copy number variation was detected

The neural network analysis identified genes significantly associated with ulceration as 'influencers' or 'influenced': and the networks generated to show the 500 most significant interactions in ulcerated and non-ulcerated tumours are shown in Figure S4.

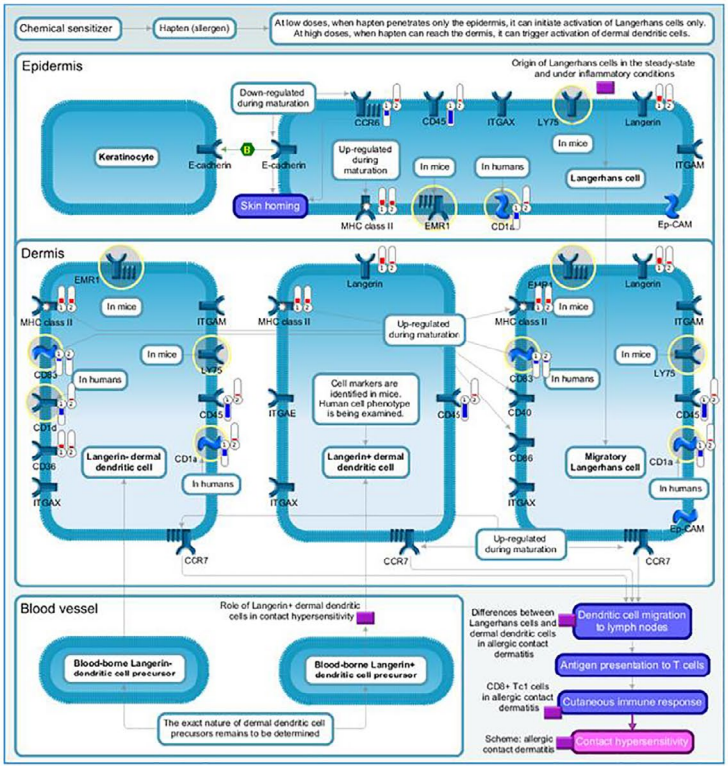
The 1000 of these genes most strongly associated with ulceration whether in the transcriptomomic or copy number data were analysed using MetaCore to identify associated biological systems in context (Figure 3). This analysis identified 21 pathways associated with differentially expressed genes, the five most significantly associated with ulceration are shown in Figure S4. The top pathway, cytoskeleton remodelling; keratin filaments, is illustrated in Figure S5a MetaCore figure (Figure S5b), it can be seen that the principal 'influenced' genes were those coding for keratins 1, 5, 14 and 16, desmoplakin and tubulin genes. Figure 3a relates also to differential gene expression of surface proteins in antigen-presenting cells in the skin, in particular dendritic cells (predominantly reduced expression of 'influenced' genes) including *CD1a* and *CCR6* but with increased expression of the genes coding for the dendritic cell marker langerin and MHC class II (Figure 3). There was strong evidence for β-catenin signalling in the significant network analysis as associated with ulceration (Figure 3b), in which expression of genes such as *Tcf (Lef)*, *BMP4*, *ITF2*, *Survivin* and *SFRP2* was identified as nodal genes.

4 | DISCUSSION

This study was designed to examine selected host factors as associated with ulceration and to further explore the tumour genomic correlates with ulceration status. The study's strengths were that two large independent data sets were available to explore host factors and the larger data set, the LMC benefitted from multi-omic tumour data from a large number of primary tumours. The proportion of participants with ulceration was 32% for the LMC and 39% in the VDI, both figures being higher than population estimates and reflect some bias in part determined by the inability to sample thin tumours and in part an intentional selection for ulceration in the VDI study. We analysed the genomic data using conventional pathway analysis tools and a neural network analysis. The weakness of this observational study is that, even with such extensive investigations, the high multidimensionality of the data preclude statistically 'conclusive' analyses; nevertheless, construction and analysis of such datasets are required to focus further research.

Ulcerated tumours were thicker and more mitotically active which is consistent with the genomic analysis, in which upregulation of biological pathways associated with cell proliferation was observed. Ulceration was also more common with increasing age in both datasets, as has been reported by others previously (Weiss et al., 2016). This was independent of thickness, indicating distinct

(a)



(b)

No	Network name	Processes	Size	Target	Pathways	p-Value	zScore	gScore
1	Tcf(Lef), TCF7L2 (TCF4), WNT, WNT3A, MITF	canonical Wnt signaling pathway (56.0%), cell-cell signal ng by wnt (64.0%), Wnt signaling pathway (64.0%), cell surface re:eptor signaling pathway involved in cell-cell signaling (66.0%), regulation of Wnt signaling pathway (60.0%)	50	11	258	2.09e-16	23.65	346.15

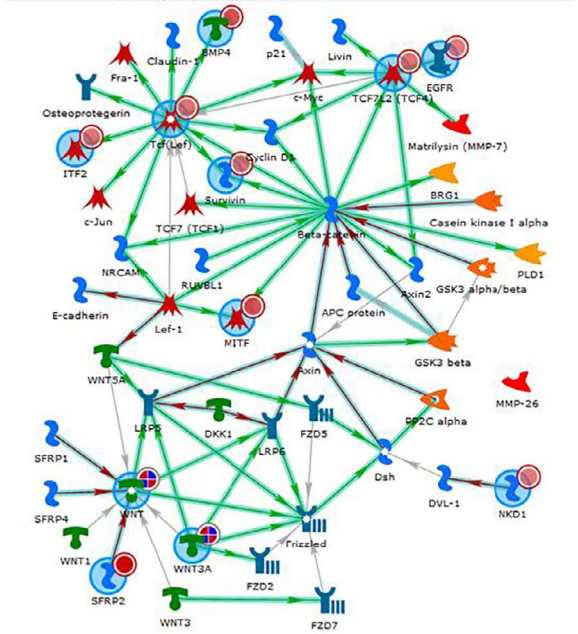


FIGURE 3 MetaCore™ version 20.1 (Clarivate Analytics, Clarivate™) pathway analysis of the 1000 genes identified using a neural network analysis of differentially expressed genes and copy number changes in tumour samples, as influencers or as influenced in ulcerated tumours compared with non-ulcerated tumours. (a) Illustrates the second most significantly associated pathway 'Populations of skin dendritic cells involved in contact hypersensitivity'. Positive differences between ulcerated and non-ulcerated are shown as red thermometers, negative differences as blue. Influenced genes are shown in (1) and influencer genes in (2). The most significantly associated pathway is illustrated in supplementary Figure S5. (b) The significantly associated network (WNT-β-catenin signalling). Thick cyan lines indicate the fragments of canonical pathways. Genes with positive differences between ulcerated and non-ulcerated are marked with red circles; negative differences with blue circles. The 'checkerboard' colour indicates mixed expression for the gene between files or between multiple tags for the same gene. Nodal genes are highlighted by cyan circles

biological processes. There are genomic differences in melanomas with age, tumours with driver mutations in *BRAF* being more common in the young. It is also, however, well recognised that survival expectation is reduced in older melanoma patients putatively related to reduced immune function (Weiss et al., 2016) and/or to differing tumour microenvironments (Fane & Weeraratna, 2020; Wellbrock, 2016), so whether tumour or host variation explains the increased ulceration frequency in older people is not clear.

That ulceration was much more frequent in tumours arising in sun-protected body sites, for example the genitalia and acral sites, was of note: these body sites are subject to friction and it is therefore possible that trauma might play an aetiological role in ulceration. However, since ulceration was significantly less frequent in melanomas of the arms and legs (which one argues are also subject to trauma) suggests that the site variation might be biological rather than related to trauma.

Here, we report that the neural network analysis identified Wnt/β-catenin signalling as associated with ulceration, a pathway known to be associated with reduced host immune responses to melanoma (Spranger et al., 2015). We have recently reported that vitamin D/VDR signalling drives suppression of Wnt/β-catenin signalling in an in vivo B16 model (Muralidhar et al., 2019) and that *VDR* expression was lower in patient tumours arising in sun-protected sites. We hypothesise that ulceration may be more common in tumours arising in those sites as a manifestation of low VDR expression and consequent increased β-catenin signalling, but further investigation of this is beyond the scope of this paper.

We had previously reported that ulceration was associated with vitamin D deficiency and smoking in a multivariable analysis of the larger LMC data set (Jewell et al., 2015), and here, we report that in the significantly smaller VDI study the data were consistent with an increased risk of ulceration in vitamin D deficiency. Finally, with reference to host factors postulated to be biologically associated with ulceration, although we saw no significant association between individual circulating inflammatory markers and ulceration status in either the LMC or VDI study, we report that the GPS (high CRP and low albumin levels) was associated with poorer survival in the LMC (the association driven primarily by albumin levels). Taken together, we argue that these data support the hypothesis that host factors play a significant role in host tumour interaction in melanoma

and consequent survival expectation rather than ulceration per se. Vitamin D levels are negatively correlated with CRP (Amer & Qayyum, 2012), and vitamin D is reported to have anti-inflammatory effects in oncogenesis, recently reviewed (Liu et al., 2018). These effects of vitamin D are reported to be mediated by diverse mechanisms including reduced levels of pro-inflammatory cytokines (such as IL-6) and inhibition of pro-inflammatory mediators including the prostaglandin/COX2 pathway (Liu et al., 2018). Conversely, the ill effects of smoking on health, such as cardiovascular disease, are mediated in part by the induction of chronic systemic inflammation. Although we did not see any significant relationship between the ulceration phenotype and circulating hsCRP or fibrinogen in the VDI, the data were consistent with a relationship for fibrinogen (OR for ulceration = 1.58, $p = .1$) given the smaller size of the data set. The differences in designs of the two studies preclude pooling the data and producing joint analyses; however, consistency in direction and magnitude of effects is reassuring and suggests observations worth further investigation. Our study provides further evidence, albeit indirect, for a role of systemic inflammation in death from melanoma. We report evidence that brisk tumour-infiltrating lymphocytes in the LMC (as reported by clinical histopathology review) were less likely in people with lower albumin levels, and in univariable analyses at least, in people with ulcerated primaries, suggests that systemic inflammation/ill health has the net effect of reducing adaptive immune responses to cancer, although this was not seen in the significantly smaller VDI data set.

In terms of understanding the biological significance of ulceration, the single observer histopathology analysis of the area of the invasive tumour from which the FFPE core was taken for genomic studies provided supportive evidence that ulcerated tumours are immunologically impaired as they had fewer lymphocytes and macrophages but more neutrophils. We did not validate our previous observation of increased macrophages in ulcerated tumours in an immunohistochemical study (Storr et al., 2012), but the current observations were based upon morphology alone. These observations are consistent with the concept of a protumourigenic environment. We also report differential expression of cytokine and chemokine genes and skewed infiltration of immune cell types in ulcerated tumours. Ulcerated tumours had a relative deficit of cytokine gene expression and there was evidence for lower signals from 15 inferred immune cell subsets in ulcerated tumours, including dendritic, T and NK cells, all of which have established anti-tumour activity. This is taken as further evidence that anti-tumour immune responses to melanomas are lower in ulcerated tumours, and this is likely to explain at least in part, the associated worse prognosis. The 13 upregulated cytokine genes included those coding for IL1β and IL6, both key pro-inflammatory cytokines. Active IL-1β is produced by inflammasome activation in macrophages and results in increased levels of IL-6 which stimulates production of acute phase proteins, including CRP (Ridker, 2016; Ridker et al., 2012). Chronic activation of the pathway systemically is deleterious and mediates a range of pathologies, including cardiovascular disease. The CANTOS trial established that IL-1β inhibition reduced further cardiovascular events after myocardial infarction (Ridker et al., 2018). Notably, the CANTOS trial also provided evidence of reduced lung cancer deaths in participants randomised to active treatment (Ridker et al., 2017). The resultant hypothesis is that inflammation mediated by IL-1β signalling plays a significant role in cancer and our findings reported here are supportive of a role of that signalling pathway in ulcerated tumours. The analysis of results from immunotherapy trials has not to our knowledge shown evidence for an interaction with ulceration status but this would be sensible to explore. We tested the hypothesis that systemic inflammation contributes to the development of microscopic ulceration by asking if microscopic ulceration predicted deaths from causes other than melanoma, and the data were consistent with this, and indeed as postulated, the overwhelming causes of death were from diseases known to be mediated by systemic inflammation.

We report evidence of a relative increase in neutrophils and reduced T cells in the single observer histopathological analysis of ulcerated tumours, broad components of the innate and adaptive immune cells being reduced in ulcerated tumours in the inferred immune cell analysis. Increased numbers of neutrophils have been observed previously in ulcerated melanomas, although functional studies suggested that the neutrophils were contributing to necrosis rather than tumour progression (Schedel et al., 2020). We saw upregulation of the gene coding for CXCL5 in the transcriptomes of ulcerated tumours and this cytokine is reported to be increased in thicker primaries, to facilitate melanoma cell-neutrophil interaction (Soler-Cardona et al., 2018), and we speculate that pro-inflammatory cytokines in ulcerated tumours may be associated with neutrophil-mediated protumourigenic inflammation. Soler-Cardona et al. (2018) argued that their data suggest that neutrophils travel from melanomas to regional nodes where they prepare the way for metastases. The apparent reduction in other immune cells such as macrophages in ulceration despite the positive relationship between ulceration and the presence of regression may reflect a sampling bias in that the presence of regression was determined by examination of the whole slide whereas the transcriptomic examination was of a core taken from the advancing edge of the tumour.

Unexpectedly, the mutation load was lower in ulcerated tumours, especially as thicker tumours tend to have higher numbers of mutations, although this was not statistically significant. This finding is unexplained but may reflect bias related to the site of the original tumour as ulceration was more frequent in tumours arising in sun-protected sites which commonly have fewer mutations and do not consistently have the UV signature of C>T (Rawson et al., 2017). The measure of mutation load derived was based upon targeted sequencing of candidate genes rather than whole-genome sequencing, but nonetheless, mutation load was positively associated with better survival, as has been reported particularly in relation to passenger mutations (Klebanov et al., 2019) and is thought to reflect greater antigenicity. *BRAF*- and *RAC1*-mutated tumours have been reported to be associated with ulceration (Mar et al., 2014; Spathis et al., 2019) but we saw no evidence for that (*BRAF* $p = .34$ and *RAC 1* $p = .3$) and indeed the evidence for association between

any specific mutation and ulceration status was weak. The two most persuasive results were that mutations in the gene coding for p53 were associated with ulceration while those in APC were protective. There is a strong evidence base that mutant p53 plays a role in pro-inflammatory mechanisms in cancer (Cooks & Harris, 2014; Ham et al., 2019) and that wild type p53 has an important function in immune responses to cancer (Blagih et al., 2020). It is therefore possible that in the 21 tumours with p53 mutations, the genotype may have contributed to the ulceration phenotype. Loss of p53 has previously been reported to result in activation of Myc in breast cancer (Santoro et al., 2019) and this observation is therefore consistent with the transcriptomic data reported here in which the Wnt/β-catenin pathway was shown to be associated with ulceration. The tumour suppressor APC is a component of the β-catenin destruction complex (Hankey et al., 2018), and APC mutations are thus predicted to increase β-catenin levels, a known contributor to melanoma progression (Nsengimana et al., 2018; Spranger et al., 2015). Why APC mutations are counterintuitively protective for ulceration and why higher levels of APC gene expression are associated with a poorer survival is therefore not understood in this context, although the number of tumours with mutated *APC* was small and this therefore may be a chance finding. However, a β-catenin independent action of APC (Hankey et al., 2018) might regulate ulceration, for example the APC protein is known to play a part in multiple complexes at the cell surface and to play a role in cell adhesion (van Es et al., 2001).

The agnostic analysis of copy number data did not identify evidence for a specific deletion or amplification driving ulceration (an association which survived correction for multiple testing). However, the pathway analysis suggests that copy number changes in genes involved in cytoskeleton remodelling, in particular increased copy number of the gene coding for tubulin beta (*TUBB2B*) at 6p25.2 was associated with ulceration. Many of these amplifications were extensive in keeping with the known common cytogenetic profile of melanoma. It is of note that the gene is close to *RREB1* and that amplification at 6p is common in melanoma. Amplification of *RREB1* specifically has previously been reported in acral lentiginous melanoma in one study using FISH in 3/3 acral tumours (Gerami et al., 2010), and in a second, 23 of 44 cases (Su et al., 2017) and it is of note that acral lentiginous tumours are often ulcerated. The computed fraction of genome altered by copy number change was higher in ulcerated tumours in contrast to mutation load which probably reflects the dominance of rare melanomas in the ulcerated tumour subgroup, in that these rare subtypes are reported to be driven by copy number (Bastian et al., 2000). Plakophilin 1 (*PKP1*) is reported to be a component of desmosomes but one which can play a role in modulating the balance between stability and proliferation and can be expressed by some melanomas (Wolf et al., 2013). More recently, a network analysis identified *PKP1* (coding for ras responsive element binding protein 1) as a key melanoma hub gene, but loss of expression was associated with poorer outcomes whereas our analysis suggested that amplification was associated with ulceration (Wang, Wang, et al., 2019). Examination of the traces showed that amplification of the gene coding for plakophilin at 1q32.1 was most frequently a part of a large amplification of the long arm of chromosome 1, at which the genes coding for Jun and L-Myc lie, and selection for these oncogenes rather than *PKP1* may have been the selective force. These observations suggest that extensive copy number changes on 6p and 1q well known to occur in melanoma may play a genomic role in determining the ulceration phenotype.

The neural network analysis shows that a number of keratin genes were identified as negatively influenced in ulcerated tumours. These included keratins 5 and 14 which code for proteins which form a keratin pair expressed in basal keratinocytes and are less expressed as the epidermis differentiates (Alam et al., 2011). It is assumed that the keratin 5 and 14 signals derive from epidermal structures included in the process of sampling melanomas, and therefore that in this neural network analysis, this signal derives from the transcriptomic (rather than the copy number) data. We postulate that these 'influenced' epidermal genetic differences are driven by other genomic changes, resulting in reduced expression of keratins expressed most strongly in basal cells contributing to lack of adherence and therefore ulceration. While the TMA cores were not intended to sample the epidermis, it was not possible always to avoid hair follicles for example. The copy number data identified changes in genes coding for other proteins involved in cytoskeletons and our hypothesis is that these copy number changes were more likely to have occurred in the tumour cells (although a very recent paper has reported copy number changes in stromal cells of colon cancer using single cell genomics (Zhou et al., 2020), and may be drivers of the ulceration process. It is of note that analysis of the copy number data alone identified the same cytoskeleton remodelling pathways as did the neural network analysis, albeit different genes (Figures S2 and S5), consistent with the view that this is a key pathway associated with ulceration and that both copy number and transcriptomic changes drive this. The second pathway identified influenced genes coding for surface markers of immune cells, notably epidermal Langerhans cells (dendritic cells) as characteristic of ulcerated tumours, including negatively associated pan leukocyte marker CD45, CD83 [activated T cells and dendritic cells (Ju et al., 2016)] and members of the CD1 family. These findings are consistent with the histological evidence of reduced lymphocytes in ulcerated tumours and a reduction in cytokine gene expression associated with adaptive immunity.

That the neural network analysis identified Wnt/β-catenin signalling as associated with ulceration is consistent with previous literature identifying the pathway as pro-proliferative (Muralidhar et al., 2019; Wang et al., 2019). The nodal genes (all positively associated with ulceration) included those coding for TCF, survivin, MITF, NKD1, SPRP2 and EGF4, but the *β-catenin* gene was at the centre of the network. This is consistent with an immunosuppressive environment as strong evidence has accumulated in recent years that this pathway attenuates anti-tumoural immunity (Li et al., 2019; Spranger et al., 2015) (Nsengimana et al., 2018) and plays a significant part in secondary resistance to immunotherapy (Trujillo et al., 2019). That we report evidence that Wnt/β-catenin signalling plays a significant role in ulceration is also consistent with our reported observation that ulceration was associated with

vitamin D deficiency in the LMC (Newton-Bishop et al., 2015) and that our genomic studies identified Wnt/β-catenin signalling as upregulated in the presence of reduced vitamin D/vitamin D receptor signalling in primary melanoma (Muralidhar et al., 2019). Taken together, the genomic observations in this study are consistent with perturbed immune responses in ulcerated tumours and that the ulceration phenotype is at least in part mediated by reduced expression of key keratin filaments, which play a role in epidermal cohesion and adhesion to the dermis. The individual genes indicated by the genomic data as 'drivers' of ulceration are p53 and those coding for a number of cytoskeletal proteins such as plakophilin, but Wnt/β-catenin signalling appeared to be playing a key role in the phenotype.

One of the motivations for our study was to better understand ulceration as a putative marker of more benefit from adjuvant interferon. Interferon treatment is reported to have pleotropic effects *in vivo*, but in particular to increase STAT1 and reduce STAT3 (Ascierto & Kirkwood, 2008). We hypothesise that melanoma cells from tumours with microscopic ulceration may respond better to adjuvant interferon as a result of reversal of STAT1/3 imbalance (protumourigenic inflammation) following adjuvant interferon. Whether tumourigenic inflammation is a direct action of IL-1β, or mediated by pathways downstream of this cytokine (such as the induction of IL-6) is unclear.

In conclusion, in this study we report evidence that microscopic ulceration of primary melanoma is associated with some host factors such as older age, vitamin D deficiency and smoking, all of which are associated with chronic activation of IL-1β signalling. The tumour analyses provide evidence that ulcerated melanomas are thicker and more mitotically active which is well known, and here, we report that there was also greater vascular invasion. There was evidence or reduced adaptive immunity in terms of reduced TILs in ulcerated tumours, more neutrophils and perturbation of cutaneous immune function on pathway analysis of the transcriptomic data. Differential expression of cytokine genes was consistent with the hypothesis that although ulceration is associated with reduced TILs, that there was an inflammatory state in the tumours associated with IL1-β signalling indicating a shift in the inflammatory balance towards tumuorigenicity. There was evidence from the pathway analysis that the key associated pathway was β-catenin signalling. This pathway plays a significant role in cell proliferation in melanoma and immunosuppression (Trujillo et al., 2019) and is modified by vitamin D/vitamin D receptor signalling (Muralidhar et al., 2019), and it is of note that it is also reported to activate the inflammasome (Huang et al., 2020).

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CONFLICT OF INTERESTS

The authors declare that they have no conflict of interest.

AUTHOR CONTRIBUTIONS

John Davies: Data curation, formal analysis, investigation, validation, and writing review and editing. **Sathya Muralidhar:** Conceptualisation, formal analysis, data curation, investigation, and writing review and editing. **Juliette Randerson-Moor:** Data curation, investigation, methodology, visualisation, resources/, and writing review and editing. **Sally O'Shea and Joey Diaz:** Data curation, investigation, resources, and writing review and editing. **Christy Walker:** Data curation, and writing review and editing. **Jerémie Nsengimana:** Investigation, and writing review and editing. **Jon Laye:** Data curation, resources, and writing review and editing. **Tracey Mell:** Data curation, resources, and writing review and editing. **May Chan:** Data curation, and writing review and editing. **Lizzie Appleton:** Formal analysis, investigation, and writing review and editing. **Mark Harland:** Data curation, investigation, resources, and writing review and editing. **Sofia Chen:** Data curation, investigation, resources, and writing review and editing. **David J. Adams:** Conceptualisation, data curation, investigation, resources, and writing review and editing. **Graham P. Cook:** Conceptualisation, investigation, and writing review and editing. **Graham Ball:** Conceptualisation, data curation, investigation, resources, and writing review and editing. **David T. Bishop:** Conceptualisation, data curation, funding acquisition, investigation, resources, and writing review and editing. **Julia Newton-Bishop:** Conceptualisation, data curation, funding acquisition, investigation, resources, writing original draft, and writing review and editing.

DATA AVAILABILITY STATEMENT

The data sets produced in this study are available in the following databases: Transcriptomic data—Name of repository: European Genome-phenome Archive (EGA; https://ega-archive.org), Accession number: EGAS00001002922. Mutation data—European Genome-phenome Archive (EGA), Accession number: EGAS00001002409. Computer code Code used in the Neural Network Analysis licensed by Intelligent Omics, Biocity Nottingham https://www.intellomx.com.

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REFERENCES

Abdel-Fatah, T. M. A., Agarwal, D., Liu, D.-X., Russell, R., Rueda, O. M., Liu, K., Xu, B., Moseley, P. M., Green, A. R., Pockley, A. G., Rees, R. C., Caldas, C., Ellis, I. O., Ball, G. R., & Chan, S. Y. T. (2016). SPAG5 as a prognostic biomarker and chemotherapy sensitivity predictor in breast cancer: a retrospective, integrated genomic, transcriptomic, and protein analysis. *The Lancet Oncology*, *17*(7), 1004–1018. https://doi.org/10.1016/S1470-2045(16)00174-1

Alam, H., Sehgal, L., Kundu, S. T., Dalal, S. N., & Vaidya, M. M. (2011). Novel function of keratins 5 and 14 in proliferation and differentiation of stratified epithelial cells. *Molecular Biology of the Cell*, *22*(21), 4068–4078. https://doi.org/10.1091/mbc.E10-08-0703

Amer, M., & Qayyum, R. (2012). Relation between serum 25-hydroxyvitamin d and C-reactive protein in asymptomatic adults (from the continuous national health and nutrition examination survey 2001 to 2006). *The American Journal of Cardiology*, *109*(2), 226–230. https://doi.org/10.1016/j.amjcard.2011.08.032

Angelova, M., Charoentong, P., Hackl, H., Fischer, M. L., Snajder, R., Krogsdam, A. M., Waldner, M. J., Bindea, G., Mlecnik, B., Galon, J., & Trajanoski, Z. (2015). Characterization of the immunophenotypes and antigenomes of colorectal cancers reveals distinct tumor escape mechanisms and novel targets for immunotherapy. *Genome Biology*, *16*, 64. https://doi.org/10.1186/s13059-015-0620-6

Arbiser, J. L. (2014). PHIPing out: A genetic basis for tumor ulceration. *The Journal of Investigative Dermatology*, *134*(3), 600–602. https://doi.org/10.1038/jid.2013.443

Ascierto, P. A., & Kirkwood, J. M. (2008). Adjuvant therapy of melanoma with interferon: Lessons of the past decade. *Journal of Translational Medicine*, *6*, 62. https://doi.org/10.1186/1479-5876-6-62

Bastian, B. C., Kashani-Sabet, M., Hamm, H., Godfrey, T., Moore, D. H. 2nd, Brocker, E. B., & Pinkel, D. (2000). Gene amplifications characterize acral melanoma and permit the detection of occult tumor cells in the surrounding skin. *Cancer Research*, *60*(7), 1968–1973. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=10766187

Blagih, J., Buck, M. D., & Vousden, K. H. (2020). p53, cancer and the immune response. *Journal of Cell Science*, *133*(5), https://doi.org/10.1242/jcs.237453

Cooks, T., & Harris, C. C. (2014). p53 mutations and inflammation-associated cancer are linked through TNF signaling. *Molecular Cell*, *56*(5), 611–612. https://doi.org/10.1016/j.molcel.2014.11.018

Fane, M., & Weeraratna, A. T. (2020). How the ageing microenvironment influences tumour progression. *Nature Reviews Cancer*, *20*(2), 89–106. https://doi.org/10.1038/s41568-019-0222-9

Filia, A., Droop, A., Harland, M., Thygesen, H., Randerson-Moor, J., Snowden, H., Taylor, C., Diaz, J. M. S., Pozniak, J., Nsengimana, J., Laye, J., Newton-Bishop, J. A., & Bishop, D. T. (2019). High-resolution copy number patterns from clinically relevant FFPE material. *Scientific Reports*, *9*(1), 8908. https://doi.org/10.1038/s41598-019-45210-2

Gao, J., Shi, L. Z., Zhao, H., Chen, J., Xiong, L., He, Q., & Sharma, P. (2016). Loss of IFN-gamma pathway genes in tumor cells as a mechanism of resistance to anti-CTLA-4 therapy. *Cell*, *167*(2), 397–404.e9. https://doi.org/10.1016/j.cell.2016.08.069

Gerami, P., Mafee, M., Lurtsbarapa, T., Guitart, J., Haghighat, Z., & Newman, M. (2010). Sensitivity of fluorescence in situ hybridization for melanoma diagnosis using RREB1, MYB, Cep6, and 11q13 probes in melanoma subtypes. *Archives of Dermatology*, *146*(3), 273–278. https://doi.org/10.1001/archdermatol.2009.386

Ham, S. W., Jeon, H.-Y., Jin, X., Kim, E.-J., Kim, J.-K., Shin, Y. J., Lee, Y., Kim, S. H., Lee, S. Y., Seo, S., Park, M. G., Kim, H.-M., Nam, D.-H., & Kim, H. (2019). TP53 gain-of-function mutation promotes inflammation in glioblastoma. *Cell Death and Differentiation*, *26*(3), 409–425. https://doi.org/10.1038/s41418-018-0126-3

Hankey, W., Frankel, W. L., & Groden, J. (2018). Functions of the APC tumor suppressor protein dependent and independent of canonical WNT signaling: Implications for therapeutic targeting. *Cancer and Metastasis Reviews*, *37*(1), 159–172. https://doi.org/10.1007/s10555-017-9725-6

Huang, L., Luo, R., Li, J., Wang, D., Zhang, Y., Liu, L., & Zhao, K. (2020). Beta-catenin promotes NLRP3 inflammasome activation via increasing the association between NLRP3 and ASC. *Molecular Immunology*, *121*, 186–194. https://doi.org/10.1016/j.molimm.2020.02.017

Jewell, R., Elliott, F., Laye, J., Nsengimana, J., Davies, J., Walker, C., Conway, C., Mitra, A., Harland, M., Cook, M. G., Boon, A., Storr, S., Safuan, S., Martin, S. G., Jirström, K., Olsson, H., Ingvar, C., Lauss, M., Bishop, T., … Newton-Bishop, J. (2015). The clinicopathological and gene expression patterns associated with ulceration of primary melanoma. *Pigment Cell Melanoma Res*, *28*(1), 94–104. https://doi.org/10.1111/pcmr.12315

Ju, X., Silveira, P. A., Hsu, W. H., Elgundi, Z., Alingcastre, R., Verma, N. D., & Hart, D. N. (2016). The analysis of CD83 expression on human immune cells identifies a unique CD83+-activated T cell population. *The Journal of Immunology*, *197*(12), 4613–4625. https://doi.org/10.4049/jimmunol.1600339

Klebanov, N., Artomov, M., Goggins, W. B., Daly, E., Daly, M. J., & Tsao, H. (2019). Burden of unique and low prevalence somatic mutations correlates with cancer survival. *Scientific Reports*, *9*(1), 4848. https://doi.org/10.1038/s41598-019-41015-5

Koelblinger, P., Emberger, M., Drach, M., Cheng, P. F., Lang, R., Levesque, M. P., & Dummer, R. (2019). Increased tumour cell PD-L1 expression, macrophage and dendritic cell infiltration characterise the tumour microenvironment of ulcerated primary melanomas. *Journal of the European Academy of Dermatology and Venereology*, *33*(4), 667–675. https://doi.org/10.1111/jdv.15302

Li, X., Xiang, Y., Li, F., Yin, C., Li, B., & Ke, X. (2019). WNT/beta-catenin signaling pathway regulating T cell-inflammation in the tumor microenvironment. *Frontiers in Immunology*, *10*, 2293. https://doi.org/10.3389/fimmu.2019.02293

Liu, W., Zhang, L., Xu, H. J., Li, Y., Hu, C. M., Yang, J. Y., & Sun, M. Y. (2018). The anti-inflammatory effects of vitamin D in tumorigenesis. *International Journal of Molecular Sciences*, *19*(9), https://doi.org/10.3390/ijms19092736

Mar, V. J., Wong, S. Q., Logan, A., Nguyen, T., Cebon, J., Kelly, J. W., & McArthur, G. A. (2014). Clinical and pathological associations of the activating RAC1 P29S mutation in primary cutaneous melanoma. *Pigment Cell Melanoma Research*, *27*(6), 1117–1125. https://doi.org/10.1111/pcmr.12295

Mascaro, J. M., Castro, J., Castel, T., Lecha, M., Gratacos, R., & Mascaro, J. M. Jr (1984). Why do melanomas ulcerate? *Journal of Cutaneous Pathology*, *11*(4), 269–273. https://doi.org/10.1111/j.1600-0560.1984.tb00377.x

Muralidhar, S., Filia, A., Nsengimana, J., Pozniak, J., O'Shea, S. J., Diaz, J. M., & Newton-Bishop, J. (2019). Vitamin D-VDR signaling inhibits Wnt/beta-catenin-mediated melanoma progression and promotes antitumor immunity. *Cancer Research*, *79*(23), 5986–5998. https://doi.org/10.1158/0008-5472.CAN-18-3927

Newton-Bishop, J. A., Beswick, S., Randerson-Moor, J., Chang, Y. M., Affleck, P., Elliott, F., & Bishop, D. T. (2009). Serum 25-hydroxyvitamin D-3 levels are associated with breslow thickness at presentation and survival from melanoma. *Journal of Clinical Oncology*, *27*(32), 5439–5444. https://doi.org/10.1200/Jco.2009.22.1135

Newton-Bishop, J. A., Davies, J. R., Latheef, F., Randerson-Moor, J., Chan, M., Gascoyne, J., & Bishop, D. T. (2015). 25-Hydroxyvitamin D2 / D3 levels and factors associated with systemic inflammation and melanoma survival in the Leeds Melanoma Cohort. *International Journal of Cancer*, *136*(12), 2890–2899. https://doi.org/10.1002/ijc.29334

Nozoe, T., Matono, R., Ijichi, H., Ohga, T., & Ezaki, T. (2014). Glasgow Prognostic Score (GPS) can be a useful indicator to determine prognosis of patients with colorectal carcinoma. *International Surgery*, *99*(5), 512–517. https://doi.org/10.9738/INTSURG-D-13-00118.1

Nsengimana, J., Laye, J., Filia, A., O'Shea, S., Muralidhar, S., Pozniak, J., & Newton-Bishop, J. (2018). Beta-catenin-mediated immune evasion pathway frequently operates in primary cutaneous melanomas. *Journal of Clinical Investigations*, *128*(5), 2048–2063. https://doi.org/10.1172/JCI95351

Ohmura, H., Uchino, K., Kajitani, T., Sakamoto, N., & Baba, E. (2017). Predictive value of the modified Glasgow Prognostic Score for the therapeutic effects of molecular-targeted drugs on advanced renal cell carcinoma. *Molecular and Clinical Oncology*, *6*(5), 669–675. https://doi.org/10.3892/mco.2017.1205

Rawson, R. V., Johansson, P. A., Hayward, N. K., Waddell, N., Patch, A.-M., Lo, S., Pearson, J. V., Thompson, J. F., Mann, G. J., Scolyer, R. A., & Wilmott, J. S. (2017). Unexpected UVR and non-UVR mutation burden in some acral and cutaneous melanomas. *Laboratory Investigation*, *97*(2), 130–145. https://doi.org/10.1038/labinvest.2016.143

Ridker, P. M. (2016). From C-reactive protein to interleukin-6 to interleukin-1: Moving upstream to identify novel targets for atheroprotection. *Circulation Research*, *118*(1), 145–156. https://doi.org/10.1161/CIRCRESAHA.115.306656

Ridker, P. M., Howard, C. P., Walter, V., Everett, B., Libby, P., Hensen, J., Thuren, T., & CANTOS Pilot Investigative Group. (2012). Effects of interleukin-1beta inhibition with canakinumab on hemoglobin A1c, lipids, C-reactive protein, interleukin-6, and fibrinogen: A phase IIb randomized, placebo-controlled trial. *Circulation*, *126*(23), 2739–2748. https://doi.org/10.1161/CIRCULATIONAHA.112.122556

Ridker, P. M., Libby, P., MacFadyen, J. G., Thuren, T., Ballantyne, C., Fonseca, F., Koenig, W., Shimokawa, H., Everett, B. M., & Glynn, R. J. (2018). Modulation of the interleukin-6 signalling pathway and incidence rates of atherosclerotic events and all-cause mortality: Analyses from the Canakinumab Anti-Inflammatory Thrombosis Outcomes Study (CANTOS). *European Heart Journal*, *39*(38), 3499–3507. https://doi.org/10.1093/eurheartj/ehy310

Ridker, P. M., Macfaden, J. G., Thuren, T., Everett, B. M., Libby, P., Glynn, R. J., & CANTOS Pilot Investigative Group. (2017). Effect of interleukin-1beta inhibition with canakinumab on incident lung cancer in patients with atherosclerosis: Exploratory results from a randomised, double-blind, placebo-controlled trial. *Lancet*, *390*(10105), 1833–1842. https://doi.org/10.1016/S0140-6736(17)32247-X

Ridker, P. M., Thuren, T., Zalewski, A., & Libby, P. (2011). Interleukin-1beta inhibition and the prevention of recurrent cardiovascular events: Rationale and design of the Canakinumab Anti-inflammatory Thrombosis Outcomes Study (CANTOS). *American Heart Journal*, *162*(4), 597–605. https://doi.org/10.1016/j.ahj.2011.06.012

Salim, A., Tai, E. S., Tan, V. Y., Welsh, A. H., Liew, R., Naidoo, N., Wu, Y. I., Yuan, J.-M., Koh, W. P., & van Dam, R. M. (2016). C-reactive protein and serum creatinine, but not haemoglobin A1c, are independent predictors of coronary heart disease risk in non-diabetic Chinese. *European Journal of Preventive Cardiology*, *23*(12), 1339–1349. https://doi.org/10.1177/2047487315626547

Santoro, A., Vlachou, T., Luzi, L., Melloni, G., Mazzarella, L., D'Elia, E., Aobuli, X., Pasi, C. E., Reavie, L., Bonetti, P., Punzi, S., Casoli, L., Sabò, A., Moroni, M. C., Dellino, G. I., Amati, B., Nicassio, F., Lanfrancone, L., & Pelicci, P. G. (2019). p53 loss in breast cancer leads to Myc activation, increased cell plasticity, and expression of a mitotic signature with prognostic Value. *Cell Reports*, *26*(3), 624–638.e8. https://doi.org/10.1016/j.celrep.2018.12.071

Schedel, F., Mayer-Hain, S., Pappelbaum, K. I., Metze, D., Stock, M., Goerge, T., Loser, K., Sunderkötter, C., Luger, T. A., & Weishaupt, C. (2020). Evidence and impact of neutrophil extracellular traps in malignant melanoma. *Pigment Cell & Melanoma Research*, *33*(1), 63–73. https://doi.org/10.1111/pcmr.12818

Sepehri, Z., Masoumi, M., Ebrahimi, N., Kiani, Z., Nasiri, A. A., Kohan, F., & Asadikaram, G. (2016). Atorvastatin, losartan and captopril lead to upregulation of TGF-beta, and downregulation of IL-6 in coronary artery disease and hypertension. *PLoS One*, *11*(12), e0168312. https://doi.org/10.1371/journal.pone.0168312

Soler-Cardona, A., Forsthuber, A., Lipp, K., Ebersberger, S., Heinz, M., Schossleitner, K., Buchberger, E., Gröger, M., Petzelbauer, P., Hoeller, C., Wagner, E., & Loewe, R. (2018). CXCL5 facilitates melanoma cell-neutrophil interaction and lymph node metastasis. *The Journal of Investigative Dermatology*, *138*(7), 1627–1635. https://doi.org/10.1016/j.jid.2018.01.035

Spathis, A., Katoulis, A., Damaskou, V., Liakou, A., Kottaridi, C., Leventakou, D., Sgouros, D., Mamantopoulos, A., Rigopoulos, D., Karakitsos, P., & Panayiotides, I. (2019). BRAF mutation status in primary, recurrent, and metastatic malignant melanoma and its relation to histopathological parameters. *Dermatology Practical & Conceptual*, *9*(1), 54–62. https://doi.org/10.5826/dpc.0901a13

Spranger, S., Bao, R., & Gajewski, T. F. (2015). Melanoma-intrinsic beta-catenin signalling prevents anti-tumour immunity. *Nature*, *523*(7559), 231–235. https://doi.org/10.1038/nature14404

Storr, S. J., Safuan, S., Mitra, A., Elliott, F., Walker, C., Vasko, M. J., Ho, B., Cook, M., Mohammed, R. A. A., Patel, P. M., Ellis, I. O., Newton-Bishop, J. A., & Martin, S. G. (2012). Objective assessment of blood and lymphatic vessel invasion and association with macrophage infiltration in cutaneous melanoma. *Modern Pathology*, *25*(4), 493–504. https://doi.org/10.1038/modpathol.2011.182

Su, J., Yu, W., Liu, J., Zheng, J., Huang, S., Wang, Y., Qi, S., Ma, X., Chen, J., & Zhang, Y. (2017). Fluorescence in situ hybridisation as an ancillary tool in the diagnosis of acral melanoma: A review of 44 cases. *Pathology*, *49*(7), 740–749. https://doi.org/10.1016/j.pathol.2017.08.006

Tong, D. L., Boocock, D. J., Dhondalay, G. K., Lemetre, C., & Ball, G. R. (2014). Artificial neural network inference (ANNI): A study on gene-gene interaction for biomarkers in childhood sarcomas. *PLoS One*, *9*(7), e102483. https://doi.org/10.1371/journal.pone.0102483

Trujillo, J. A., Luke, J. J., Zha, Y., Segal, J. P., Ritterhouse, L. L., Spranger, S., & Gajewski, T. F. (2019). Secondary resistance to immunotherapy associated with beta-catenin pathway activation or PTEN loss in metastatic melanoma. *Journal of Immunotherapy of Cancer*, *7*(1), 295. https://doi.org/10.1186/s40425-019-0780-0

van Es, J. H., Giles, R. H., & Clevers, H. C. (2001). The many faces of the tumor suppressor gene APC. *Experimental Cell Research*, *264*(1), 126–134. https://doi.org/10.1006/excr.2000.5142

von Schuckmann, L. A., Smith, D., Hughes, M. C. B., Malt, M., van der Pols, J. C., Khosrotehrani, K., & Green, A. C. (2017). Associations of statins and diabetes with diagnosis of ulcerated cutaneous melanoma. *The Journal of Investigative Dermatology*, *137*(12), 2599–2605. https://doi.org/10.1016/j.jid.2017.07.836

Wang, H.-Z., Wang, F., Chen, P.-F., Zhang, M., Yu, M.-X., Wang, H.-L., Zhao, Q., & Liu, J. (2019). Coexpression network analysis identified that plakophilin 1 is associated with the metastasis in human melanoma. *Biomedicine & Pharmacotherapy*, *111*, 1234–1242. https://doi.org/10.1016/j.biopha.2018.12.135

Wang, S., Wu, Y., Xu, Y., & Tang, X. (2019). miR-10b promoted melanoma progression through Wnt/beta-catenin pathway by repressing ITCH expression. *Gene*, *710*, 39–47. https://doi.org/10.1016/j.gene.2019.05.043

Weiss, S. A., Han, J., Darvishian, F., Tchack, J., Han, S. W., Malecek, K., Krogsgaard, M., Osman, I., & Zhong, J. (2016). Impact of aging on host immune response and survival in melanoma: An analysis of 3 patient cohorts. *Journal of Translational Medicine*, *14*(1), 299. https://doi.org/10.1186/s12967-016-1026-2

Wellbrock, C. (2016). Melanoma and the microenvironment-age matters. *New England Journal of Medicine*, *375*(7), 696–698. https://doi.org/10.1056/NEJMcibr1606907

Wolf, A., Rietscher, K., Glass, M., Huttelmaier, S., Schutkowski, M., Ihling, C., & Hatzfeld, M. (2013). Insulin signaling via Akt2 switches

plakophilin 1 function from stabilizing cell adhesion to promoting cell proliferation. *Journal of Cell Science*, *126*(Pt 8), 1832–1844. https://doi.org/10.1242/jcs.118992

Zhou, Y., Bian, S., Zhou, X., Cui, Y., Wang, W., Wen, L. U., Guo, L., Fu, W., & Tang, F. (2020). Single-cell multiomics sequencing reveals prevalent genomic alterations in tumor stromal cells of human colorectal cancer. *Cancer Cell*, *38*(6), 818–828.e5. https://doi.org/10.1016/j.ccell.2020.09.015

SUPPORTING INFORMATION

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