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25-hydroxyvitamin D_2/D_3 levels and factors associated with systemic inflammation and melanoma survival in the Leeds Melanoma Cohort

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Abstract

Lower 25-hydroxyvitamin D_2/D_3 levels at melanoma diagnosis are associated with thicker primaries and poorer survival. We postulated that this might relate to the deleterious effect of systemic inflammation as 25-hydroxyvitamin D_2/D_3 levels are inversely associated with levels of C-reactive protein. 2182 participants in the Leeds Melanoma Cohort (median follow up 7.98 years) provided data on drug exposure, co-morbidities and a serum 25-hydroxyvitamin D_2/D_3 level at recruitment. Factors reported to modify systemic inflammation (low vitamin D levels, high body mass index (BMI), use of aspirin or non-steroidal anti-inflammatory drugs or smoking were tested as predictors of microscopic ulceration (in which primary tumours are inflamed) and melanoma specific survival (MSS). Ulceration was independently associated with lower 25-hydroxyvitamin D_2/D_3 levels (OR=0.94 per 10nmol/L, 95% CI 0.88–1.00, p=0.05) and smoking at diagnosis (OR=1.47, 95% CI 1.00–2.15, p= 0.04). In analyses adjusted for age and sex, a protective effect was seen of 25-hydroxyvitamin D_2/D_3 levels at diagnosis on melanoma death (OR=0.89 per 10nmol/L, 95% CI 0.83–0.95, p<0.001) and smoking increased the risk of death (OR=1.13 per 10 years, 95% CI 1.05–1.22, p=0.001). In multivariable analyses (adjusted for tumour thickness) the associations with death from melanoma were low 25-hydroxyvitamin D_2/D_3 level at recruitment (<20 nmol/L vs. 20–60 nmol/L, HR=1.52, 95% CI 0.97–2.40, p=0.07) and smoking duration at diagnosis (HR=1.11, 95% CI 1.03–1.20, p=0.009). The study shows evidence that lower vitamin D levels and smoking are associated with ulceration of primary melanomas and poorer MSS. Further analyses are necessary to understand any biological mechanisms that underlie these findings.

Keywords

Melanoma; inflammation; vitamin D; survival; smoking

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Introduction

In 2009, we reported evidence that lower serum 25-hydroxyvitamin D_2/D_3 levels at diagnosis to be independently associated with poorer outcome from melanoma in the Leeds Melanoma Cohort[1]. As vitamin D levels are higher in fitter, healthier individuals[2], the relationship between vitamin D levels and melanoma survival has not yet been established to be causal: Vitamin D levels might merely be a marker of healthier lifestyles. *In vitro* studies have shown evidence that vitamin D has an anti-proliferative effect on a proportion of melanoma cells in culture[3]. Vitamin D has pleotropic effects *in vivo*, however, and importantly is a regulator of innate and adaptive immunity playing a role in the suppression of inflammation[4]. We report here an evaluation of the potential role of systemic inflammation (as evidenced by associated factors such as aspirin usage and smoking) and its moderation by vitamin D in melanoma survival.

The role of tumor related inflammation has been recognised as fostering a number of the "Hallmarks" of cancer as well as a process which can be targeted therapeutically[5]. Whilst spontaneous immunological responses to melanoma are known to occur[6], and there is unequivocal evidence that immunologically mediated long term survival can be induced therapeutically[7], there is a growing body of evidence that excessive inflammation promotes cancer metastasis[8] in part by suppressing adaptive immunity. Balkwill and colleagues[9] have suggested that "smouldering" inflammation aids proliferation and survival of cancer cells, promotes angiogenesis and metastasis, subverts immune responses and alters responses to hormones and therapeutic agents. They also postulated that cancer and inflammation are connected by two pathways: intrinsic (activated by genetic events in the tumor itself) and extrinsic (inflammation in the tumor microenvironment).

There is clear evidence in melanoma that tumors drive inflammation (the intrinsic pathway). Driver mutations such as those in *BRAF*[10] increase secretion of pro-inflammatory cytokines such as IL-6 and IL-8. There is also evidence in ulcerated tumors (ulceration is a potent poor prognostic factor for melanoma), that gene expression patterns associated with inflammation are increased[11]. In our own data, gene expression patterns associated with a "wound healing" response were associated with tumor ulceration (Jewell et al. accepted 2014). That ulceration is associated with increased blood vessel density and increased macrophage infiltration[12] suggests that ulceration may be a histopathological marker of considerable tumor-associated inflammation.

Inflammatory responses to melanoma in its microenvironment are therefore postulated to result from oncogenic changes in the tumors themselves and the responsive stromal changes, but in this study we have explored the possibility that systemic inflammation might also play a role in driving tumor progression. We have used data and samples from participants in the Leeds Melanoma Cohort[1].

Systemic inflammation in the obesity related chronic inflammatory syndrome (sometimes referred to as the "metabolic syndrome") has been reported to increase the risk of cancers other than melanoma such as colorectal cancer[13] and post menopausal breast cancer[14]. This

syndrome is also associated with lower vitamin D levels[15–17]. In fact serum levels of C-reactive protein (CRP) and 25-hydroxyvitamin D_2/D_3 are strongly negatively correlated[18]. There is also some support for the view that vitamin D may at least in part moderate the deleterious effects of obesity in terms of the metabolic syndrome[19, 20] and related immunological parameters[21, 22].

In order to explore the hypothesis that potentially modifiable systemic inflammation reduces survival expectation for melanoma patients we looked for indirect evidence in the form of an association between factors reported to reduce inflammation such as use of non-steroidal anti-inflammatories and aspirin, and vitamin D. We also looked for an association between co-morbidities associated with systemic inflammation such as diabetes, obesity and smoking in participants in the Leeds Melanoma Cohort. We then investigated the associations between these factors and tumor ulceration at presentation as tumor ulceration has been reported to be associated with inflammatory gene expression signatures in the tumor[11] (Jewell et al accepted 2014).

Material and Methods

Study design

Supplementary figure 1 shows a schematic of the study design. 2182 melanoma patients were recruited to the Leeds Melanoma Cohort in the period 2001–2013. Patients were ascertained from pathology and clinical registers in a geographically defined area of the Northern part of the UK, with additional recruitment from 32 other clinical centres carrying out sentinel node biopsy (total 342 recruits) and of rare subtypes of cases with melanomas arising in sun-protected sites (total 76 recruits). Patients were invited to participate at 3 months after diagnosis with the intent of interviewing and sampling them within the period 3 to 6 months after diagnosis. Patients responded variably quickly and the median time to interview was 5.2 months. Each participant completed detailed questionnaires, which included questions about their drug usage history, concurrent illnesses and BMI at recruitment. 25-hydroxyvitamin D_2/D_3 levels were measured in the serum sample obtained at entry into the study except for in 220 for whom no initial sample was given, 173 samples still to be processed from the most recent recruits and 5 individuals for whom the vitamin D assay failed. The Breslow thickness and ulceration status of the primary tumor were obtained from histopathology reports. 35 participants with a nodal presentation and no obvious primary were excluded from this study. Concentrations of 25-hydroxyvitamin vitamin D_2 and D_3 (nmol/L) were measured in 100 uL of cryopreserved serum by liquid chromatography tandem mass spectrometry (LC-MS/MS) by the NHS laboratory in the Leeds Teaching Hospitals Trust as described previously[1]. We will henceforth refer to the levels as vitamin D levels.

Survival

Cohort members were followed up both directly (the majority of participants by annual re-contact) as well as passively by regular review of national registers and medical records. For those cases who have died, the death certificate (or cause of death data taken from the death certificate obtained from the Office of National Statistics (ONS)) and medical records were

obtained. Melanoma specific survival (MSS) data was generated by research nurses/JNB who reviewed the evidence relating to cause of death from these sources and determined whether the cause of death for each case was melanoma related or not melanoma related. Thus a cancer registry reported cause of death was compared with medical records (primary, secondary and tertiary care) and patient reports of disease progression.

Statistical Analysis

Several variables routinely collected in the Cohort were identified as being associated or potentially associated with inflammation. These were; self reported weight and height (from which BMI was estimated) at recruitment, usage of non-steroidal anti-inflammatory drug (NSAIDs) at or in the 10 years prior to the interview date, usage of aspirin at or in the 10 years prior to the interview date, diabetes at diagnosis and smoking history. Variables for each of these measures were created from the Cohort questionnaire data. Adjusted serum vitamin D levels were generated by fitting levels in a linear model with test batch and season of blood draw (Jan-Mar, Apr-Jun, Jul-Sep, Oct-Dec) using Apr-Jun as the baseline. Vitamin D levels were classified as either deficient (less than 20 nmol/L), sub-optimal (20 to 60 nmol/L) or three levels of sufficiency. We use the 20 to 60 nmol/L category as the baseline as this was the largest group. BMI (kg/m^2) was classified using the standard classification system defined by the World Health Organisation (WHO) of underweight (<18.5), normal (18.5–25), overweight (25–30) and obese (30+)[23].

Cases were interviewed about whether they had diabetes and about their smoking habits. Four smoking-related variables were generated from these data: patient ever regularly smoked (yes/no), patient current smoker (yes/no), duration for which patient smoked (in 5 year units) and an estimate of the quantity the patient smoked in pack years based upon self reported consumption of commercial cigarettes, hand rolls, small cigars, large cigars and/or pipe tobacco (in units of 10 pack-years). Cases were also interviewed on past and current prescribed drug usage. NSAID usage was identified using the British National Formulary drug code 10.1.1 (www.BNF.org). Duration of aspirin usage was measured as the time between reported starting taking aspirin and stopping or until date of interview; cases for which no start date could be attributed were treated as missing data. Ulceration status was derived from histopathology reports: in instances where no explicit mention of ulceration was made it was assumed that the primary had not been ulcerated.

As we, and others, have previously reported an association between ulceration of the primary and histological and gene expression patterns of inflammation driven angiogenesis, we looked at (1) associations of the above listed factors with reported tumor ulceration status and subsequently (2) the association with MSS. For ulceration, we fitted logistic regression models for each factor individually adjusting for age at diagnosis and sex. For analyses of vitamin D and BMI, separate models were fitted treating those variables as both categorical and continuous to describe overall trends as well as giving readily interpretable effect sizes for standard categories. Cox proportional hazard models were fitted on the same variables to test for an association with MSS adjusting for age and sex. We fit a second set of models additionally adjusting for site of primary (grouped as Trunk, Limbs, Head/Neck, Other) and Breslow thickness. Breslow thickness is known to be the strongest single factor

associated with survival. We have previously shown however, that low vitamin D levels are associated with thickness and we postulate therefore that inflammation and factors modifying inflammation might modify thickness as well as MSS and we therefore report analyses adjusted for and not adjusted for tumor thickness. Survival time was defined as the period between the date of surgical excision of the primary and date of death or last date of follow-up (at which point records were censored). Cases with multiple primaries and/or who had responded to the request to participate later and were therefore recruited more than 2 years after diagnosis, were excluded from survival analysis.

To determine whether significant associations of inflammation variables with outcomes of interest, were independent of each other, multivariable models including all inflammation associated variables were fitted for ulceration status of the primary and MSS as above. Variables significant at the 5% level or lower in univariable analyses were carried forward into multivariable models. Where two collinear variables can be carried forward (e.g. patient ever smoked and patient still smoking) the most statistically significant variable was taken.

Results

Demographics

Table 1 shows the distributions of sex and age of the participants at diagnosis and reported presence of factors described in the literature to modify the presence of systemic smouldering inflammation (vitamin D level, BMI, NSAID or aspirin usage, diabetes and reported smoking) in the cohort and separately for cases by tumor ulceration status. From case notes there were 426 cases with recorded ulceration, 1351 had specifically recorded that there was no ulceration, and 370 cases in which there was no record of ulceration available, 25 of these were excluded as they were nodal cases with no known primary. Individuals who had ulcerated tumours were older (median 60 years in ulcerated, 55 non-ulcerated, p=0.0001), more likely to be male (p=0.001), have a diagnosis of diabetes (p=0.002), more likely to be taking aspirin (p=0.008) (although duration of use did not vary between ulcerated and non ulcerated groups (p=1.0)), more likely to have ever smoked (p=0.04) and to have smoked for a longer duration (p=0.05) as well as having consumed more tobacco (p=0.02). However, current smoking status did not differ between cases with/without ulcerated tumors.

Association Of Putative Inflammatory Factors With Ulceration

Table 2 shows the association of the inflammation variables with ulceration of the primary melanoma adjusted for age and sex and then in multivariable analysis. In the univariable analysis, higher BMI was associated with ulceration (OR=1.17, 95% CI 1.05–1.32, p=0.007), as was diabetes (OR=1.68, 95% CI 1.03–2.73, p=0.04) and still smoking at diagnosis (OR=1.45, 95% CI 1.04–2.03, p=0.03). Higher levels of vitamin D were associated with less tumor ulceration, (OR=0.93 per 10nmol/L, 95% CI 0.88–0.99, p=0.03) but there was no protective effect of aspirin (when adjusted for age and sex) or NSAIDs.

In a multivariable model, borderline significant associations persisted for vitamin D (OR=0.94,95% CI 0.88–1.00, p=0.05) and still smoking (OR=1.47,95% CI 1.01–2.15,

p=0.04). The association between ulceration and both increased BMI and diabetes did not persist in the multivariable analysis (Table 2).

Association Of Ulceration With Melanoma Specific Survival (MSS)

Microscopic ulceration is a known predictive factor for outcome in melanoma patients confirmed in the strong association between ulceration and MSS in these data (Table 3) (HR=3.23, 95% CI 2.48–4.21, p<0.001 adjusted for age and sex). This effect persisted after taking into account Breslow thickness (HR=2.03, 95% CI 1.51–2.75, p<0.001 adjusted for age, sex, site and Breslow thickness). Table 3 shows the effect of adjusting for each of the putative inflammation variables on the association of ulceration with outcome. Adjusting for vitamin D and smoking either separately or together had a small effect on the hazard ratio (HR=3.15 for either individually, 3.07 in combination). When models were additionally adjusted for tumour site and Breslow thickness, adjusting then for vitamin D and smoking had no effect on the magnitude of the estimated hazard ratio (Table 3).

Association Of Putative Inflammatory Factors With Melanoma Specific Survival

In univariable models (Table 4) serum levels of vitamin D measured at diagnosis were seen to be associated with significantly better outcome (HR for melanoma related death 0.89 per 10nmol/L, 95% CI 0.83–0.95, p<0.001). For vitamin D levels at diagnosis stratified by laboratory definitions of sufficiency, deficient levels were associated with a HR for melanoma associated death of 2.19 (95% CI 1.43–3.34, p<0.001) in univariable analysis adjusted just for age and sex. BMI was not significantly associated with outcome when it was treated as a continuous variable (HR 1.04 per 5 units, 95% CI 0.91–1.18, p=0.6). However a significant protective effect was seen for cases in the overweight range (BMI 25–30) compared to the optimal range (BMI 18.5–25) (HR=0.70, 95% CI= 0.53–0.93,p=0.01) in this univariable analysis, but there was no trend, as this protective effect was not seen for the obese (HR=1.12 95% CI=0.85–0.52, p=0.5).

No significant associations with MSS were seen for NSAID usage, duration of aspirin use or diabetes. A history of previous smoking was associated with poorer outcome (HR=1.35, 95% CI=1.06–1.71, p=0.01). Individuals who still smoked were associated with still poorer outcome (HR=1.64, 95% CI=1.17–2.304, p=0.004). Duration of smoking was also associated with poorer outcome (HR=1.13 per 10 years, 95% CI 1.05–1.22, p=0.001) but pack years were not (HR=1.00 per 10 pack years, 95% CI 0.97–1.02,p=0.8).

In the multivariable model adjusted for age and sex (Table 4) significant associations persisted between MSS and vitamin D levels (HR=0.90 per 10nmol/L, 95% CI 0.85–0.96,p=0.002), BMI (overweight v normal range HR=0.70, 95% CI 0.52–0.95, p=0.02) and duration of smoking (HR=1.15 per 10 years, 95% CI=1.07–1.24,p<0.001).

In the models adjusted additionally for tumour site and Breslow thickness (Table 5) duration of smoking (HR=1.10 per 10 years, 95% CI=1.02–1.18,p=0.01), BMI in the overweight v optimal range (HR=0.73, 95% CI=0.55–0.97, p=0.03), vitamin D levels (measured on a continuous scale, HR=0.94 per 10 nmol/L, 95% CI 0.88–1.00, p=0.05) and deficient serum levels (<20 nmol/L) (HR=1.79 compared with individuals in the 20–60 nmol/L range, 95% CI 1.15–2.78,p=0.009) were seen to be significantly associated with outcome in univariable

analysis. A borderline association was seen for smoking at diagnosis (HR=1.38, 95% CI 0.97–1.95, p=0.07). In the multivariable model both the association with BMI (HR=0.72, 95% CI 0.53–0.98, p=0.04) and the association with duration of smoking persisted (HR=1.11 per 10 years, 95% CI 1.03–1.20, p=0.009).

Discussion

Low levels of vitamin D are associated with increased death rates in many diseases, but there is limited evidence that supplementation reduces mortality. There is evidence from a meta-analysis of independent randomized clinical trials that supplementation reduces all-cause mortality[24], but individual trials in cancer prevention have to date been unconvincing. Low vitamin D levels are more common in the obese[2, 17] and the physically inactive[2]. It is therefore possible that the many associations reported between low levels of vitamin D and a variety of diseases may simply reflect low vitamin D levels as a marker of other lifestyle factors associated with disease. Serum vitamin D levels are strongly inversely correlated with serum levels of C-reactive protein, which is generally reported to be the most robust biomarker of systemic inflammation[18]. This relationship might suggest that the associations between vitamin D and numerous diseases might represent vitamin D levels acting as a marker of inflammation rather than that vitamin D being causally related.[25] There are data however, to suggest that vitamin D may also be an active moderator of inflammation and immune processes (reviewed by Guillot[4]). Evidence for an immunological role for vitamin D includes the observation that seasonal variation in vitamin D levels is reported to be associated with variation in the human blood T cell compartment[26] and vitamin D is shown to regulate pro-inflammatory cytokine levels in healthy individuals[22]. Low vitamin D levels are associated with higher circulating pro-inflammatory cytokines in HIV positive patients[27] with increased CD4+ counts in those treated with vitamin D[28]. In rheumatoid diseases there are data to support that vitamin D reduces pro-inflammatory T cell subsets TH 1 and TH 17[29].

In the Leeds Melanoma Cohort we collected data on exposures and concurrent illnesses associated with systemic inflammation. We have therefore tested the hypothesis that obesity and diabetes might be associated with increased mortality from melanoma and investigated whether vitamin D levels were statistically independently protective. We also tested whether exposure to drugs which have been reported to reduce inflammation, might be independent predictors of reduced mortality. A weakness of the study is that the detail available on usage of drugs such as NSAIDs and aspirin was limited and we hope to improve this over time.

The first question we asked however was whether factors known to be associated with systemic inflammation were associated with primary tumour ulceration, as a marker of inflammation within the tumour. In previous studies, evidence that ulceration of primary melanoma tumors is associated with gene expression and histopathological evidence of a pro-inflammatory milieu was reported (Jewell et al accepted)[11, 12]. In this study we report that factors associated with systemic inflammation, higher BMI, diabetes and smoking were indeed predictive of ulceration of the primary tumor in univariable analysis. The relationship between BMI and diabetes did not persist as independent predictors in multivariable analysis although the odds ratios remained similar and statistical power issues may be relevant.

Given the observation that ulcerated tumors have greater blood vessel density and increased numbers of macrophages[12] and gene expression patterns associated with increased release of pro-inflammatory cytokines[11], this finding gives support to the hypothesis that systemic inflammation might play a role in determining ulceration (and implicit in that, inflammation within the tumour) and that this may in part be a biological explanation for the observation that ulceration is associated with poorer outcome for melanoma patients. Smoking increases evidence of systemic inflammation including CRP levels[3031], but a weakness of our study is that we report associations only and smoking could increase the likelihood of tumor ulceration via other mechanisms. Similarly, we can conclude good evidence that vitamin D levels are associated with reduced tumor ulceration but not that the association is causally related to reduction in inflammation. Berwick et al. previously reported that solar elastosis reported histologically in association with melanoma primaries was associated with a better outcome[32] which the authors speculated could reflect a protective effect of vitamin D or that chronic sun exposure might be associated with a biologically less aggressive tumour. We have reported previously that weekend sun exposure was associated with reduced melanoma risk[33], but found no evidence of an association between weekend sun exposure and outcome (data not shown).

We report evidence that supports the well-known association of ulceration with melanoma specific survival in our data but that also that adjustment for vitamin D, smoking and to a lesser degree NSAID usage modulates this association albeit weakly. This suggests that in part at least these factors may influence melanoma survival through having an effect on ulceration. These factors have no effect once the model is adjusted for Breslow thickness, suggesting that the effect these factors may have on ulceration is captured by thickness of the primary.

We postulated that systemic inflammation might increase melanoma related deaths but we did not show strong evidence that the presence of co-morbidities such as diabetes increased death from melanoma. We report only that diabetes was non-significantly associated with melanoma specific death, (HR death 1.50, 95% CI 0. 94–2.40, p=0.09) in a univariable analysis. We actually report evidence that overweight (but not obese) individuals had better survival than individuals in the normal weight range when survival models are adjusted for age and sex and this association persisted when the model is additionally adjusted for site and Breslow thickness, but there was no trend as the effect was not seen in the obese.

We do report however, evidence that increasing vitamin D levels were associated with lower risk of melanoma associated death in multivariable analysis, and that smoking was strongly associated with an increased risk of death. Reduced vitamin D levels and smoking are associated with increased CRP levels[34, 35] and the data are therefore consistent with the view that systemic inflammation may play a part in determining melanoma survival. The study cannot be deemed to establish a causal relationship but does suggest that vitamin D and smoking may have a role in the determination of survival from melanoma patients. At the time of writing there are a lack of adequately powered studies to test an effect of smoking on cancer outcome generally, with some support for an effect of prostate cancer[36, 37] and conflicting evidence for an effect on breast cancer[36, 38, 39].There was no significant association of NSAIDs with ulceration of the primary or with melanoma specific

death. We had limited power to assess this as currently we have data only on ever/never use of NSAIDs and only 186 individuals had taken these drugs. It is clear however that there was no support at all for aspirin as a modifier of survival in this data, although use of aspirin did seem to be associated with reduced risk of ulceration of the primary.

The weaknesses of this study are that it was an association study, and that the effects of diseases such as diabetes and agents such as vitamin D and smoking have many complicated effects on health. Although this study is large at more than 2000 cases, followed up for more than 7 years there is also insufficient statistical power to explore the small effects of multiple factors. Another weakness is that although more than half of the cases were recruited within 6 months of diagnosis for some cases there is potentially a delay in vitamin D sampling for anything up to 2 years post diagnosis. Recently diagnosed patients may well alter their UVR exposure to such an extent in this time frame that the sampled serum level is not reflective of the original level at diagnosis[40]. We therefore re-ran analyses of vitamin D in subsets of the data where the blood had been sampled no more than 1 year post diagnosis and saw no material difference in the results (data not shown) suggesting that the delays in blood sampling did not materially affect our results.

Ulceration of the primary has an impact on outcome even for stage III melanoma patients, (AJCC staging system[41]), which implies either that the ulcerated tumours are biologically different even when metastatic or that host factors affect both ulceration status and outcome. That obesity, diabetes, low vitamin D and smoking in this study were related to the presence of ulceration of primary melanomas is of note. It has been hypothesised that the presence of ulceration may be associated with suppression of adaptive immunity as a result of tumor related inflammation[9] and the multivariable analyses performed here suggest that smoking and low vitamin D levels “explained’ a small proportion of the deleterious effect of ulceration on outcome. It is possible therefore that cessation of smoking and correction of low vitamin D levels after diagnosis may be of benefit in terms of melanoma specific survival, and the data provide some support for the hypothesis that the effects of smoking and vitamin D may be mediated through inflammation. This analysis however provided little evidence that medical conditions known to be associated with systemic inflammation modified melanoma specific survival expectation, although low vitamin D and smoking remained as predictors of survival. The study therefore provided evidence more for a direct effect of vitamin D and smoking on melanoma survival than a mediation through inflammation as was postulated by Autier (2014)[25] but this needs now to be explored biochemically.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

(BMI)	body mass index
(NSAIDs)	Non-steroidal anti-inflammatory drugs
(MSS)	Melanoma specific survival
OR	odds ratio
CI	confidence intervals

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Impact

The study reports for the first time that smoking at diagnosis and lower vitamin D levels are associated with higher incidence of ulceration of the primary melanoma and poorer melanoma survival. It would be prudent to suggest to melanoma patients to cease smoking and that vitamin D depletion should be avoided, even though there is as yet no evidence that such action after diagnosis would be associated with a survival benefit. Further investigation is warranted.

Table 1

Cohort demographics in relation to whether the primary tumor was reported as being ulcerated or not

Cases with no nodal primary were excluded.

	Total*	Cases with Ulcerated Tumors	Cases without Ulcerated Tumors	P value Testing Cases with/without Ulcerated Tumor
Age at Diagnosis				**0.0001****
Median (range)	56 (17–90)	60 (22–90)	55 (17–85)	
Gender				**0.001*****
Male	924 (43.0 %)	215 (50.5%)	699 (41.2%)	
Female	1223 (57.0%)	211 (49.5%)	997 (58.8%)	
Adjusted Vitamin D* (nmol/l)				0.09***
<20	109 (6%)	27 (8%)	82 (5.8%)	
20–60	1172 (67%)	222 (66.7%)	942 (66.9%)	
60–85	394 (22.4%)	74 (22%)	315 (22.3%)	
85–100	55 (3%)	10 (3%)	44 (3.1%)	
100+	26 (1.5%)	0 (0%)	25 (1.8%)	
BMI (units)				0.06***
<18.5	17 (0.8%)	1 (0.2%)	16 (1%)	
18.5–25	799 (37.8%)	140 (33.7%)	652 (38.9%)	
25–30	854 (40.4%)	174 (41.8%)	670 (40.0%)	
30+	446 (21%)	101 (24.3%)	338 (20.2%)	
NSAIDs (BNF 10.1.1)				0.9***
Ever used	184 (8.6%)	36 (8.5%)	147 (8.7%)	
Never used	1949 (91.4%)	387 (91.5%)	1539 (91.3%)	
Aspirin				**0.008*****
Ever used	247 (12%)	65(15%)	181 (11%)	
Never used	1886 (88%)	358 (85%)	1505 (89%)	
Duration of aspirin use for users (years)				1**
Median (range)	4.7 (0.08–43.5)	4.7 (0.28–43.5)	4.7 (0.08–24.1)	
Diabetes				**0.002*****
Yes	81 (4%)	27 (6.7%)	53 (3.3%)	
No	1968 (96%)	376 (93.3%)	1570 (96.7%)	
Ever smoked				**0.04*****
Yes	926 (45%)	202 (49.5%)	715 (43.8%)	
No	1137 (55%)	206 (50.5%)	917 (56.2%)	
Still smoke (yes/no)				0.2***
Yes	243 (11.8%)	55 (13.5%)	184 (11.3%)	

	Total*	**Cases with Ulcerated Tumors**	**Cases without Ulcerated Tumors**	**P value Testing Cases with/without Ulcerated Tumor**
No	1821 (88.2%)	354 (86.6%)	1448 (89.7%)	
Duration smoked, excluding non smokers (years).				**0.05****
Median (range)	20.9 (0.1–72)	23 (0.3–58.1)	20 (0.1–72)	
Pack years (inc. rolls and cigars per 10 packyears)				**0.02****
Median (range)	1.4 (0.003–188)	1.6 (0.05–10.7)	1.4 (0.003–188)	

* Including cases with missing ulceration status

** P value calculated using a Mann Whitney U test

*** P value calculated using a chi squared test.

Table 2

Association of factors reported to modify risk of systemic inflammation with the presence of ulceration of the primary melanoma, adjusted for age and sex in univariable and then multivariable analyses

	Univariable		Multivariable (n=1688)	
Factor	**OR (95% CI)**	**p-value**	**OR (95% CI)**	**p-value**
Vitamin D per 10nmol/L *	0.93 (0.88–0.99)	0.03	0.94 (0.88–1.00)	0.05
Vitamin D categorical *				
<20nmol/l	1.44 (0.90–2.29)	0.1		
20–60 nmol/l (baseline)	1.0	-		
60–85 nmol/l	0.94 (0.70–1.27)	0.7		
85–100 nmol/l	0.94 (0.46–1.90)	0.9		
100+ nmol/L	-	-		
BMI per 5 units	1.17 (1.05–1.32)	0.007	1.10 (0.96–1.27)	0.2
BMI				
< 18.5	0.27 (0.03–2.07)	0.2		
18.5–25 (baseline)	1.0	-		
25–30	1.06 (0.82–1.36)	0.7		
30+	1.30 (0.97–1.75)	0.08		
NSAIDs (ever/never) (BNF 10.1.1)	0.83 (0.56–1.22)	0.3		
Duration of aspirin use (years)	1.01 (0.97–1.05)	0.7		
Diabetes	1.68 (1.03–2.73)	0.04	1.49 (0.85–2.60)	0.2
Smoking				
Ever smoked (yes/no)	1.14 (0.91–1.43)	0.2		
Still smoke (yes/no)	1.45 (1.04–2.03)	0.03	1.47 (1.01–2.15)	0.04
Duration (per 10 years)	1.05 (0.98–1.13)	0.2		
Pack years (inc. rolls and cigars per 10 packyears)	0.99 (0.95–1.02)	0.5		

* Vitamin D adjusted for season and batch. Given values assume that blood was drawn in spring and sampled from the first batch.

Table 3

Association Of Ulceration With MSS Adjusted For Factors Known To Be Associated With Inflammation

All given hazard ratios are for the association of ulceration with melanoma specific survival in the same subset of cases from the Leeds Melanoma Cohort, adjusted for different additional variables associated with inflammation.

	Adjusted for age and sex (n=1530)		Adjusted for age, sex and Breslow (n=1518)	
	Ulceration HR (95% CI)	p-value	Ulceration HR (95% CI)	p-value
Ulceration only	3.23 (2.48–4.21)	<0.001	2.03 (1.50–2.75)	<0.001
+Vit D*	3.15 (2.41–4.11)	<0.001	2.05 (1.51–2.78)	<0.001
+BMI	3.23 (2.48–4.21)	<0.001	2.01 (1.49–2.73)	<0.001
+NSAID	3.20 (2.46–4.18)	<0.001	2.02 (1.49–2.75)	<0.001
+Aspirin Duration	3.23 (2.48–4.21)	<0.001	2.03 (1.50–2.75)	<0.001
+Diabetes	3.23 (2.48–4.21)	<0.001	2.04 (1.50–2.77)	<0.001
+Still smoke	3.16 (2.42–4.12)	<0.001	2.01 (1.48–2.72)	<0.001
+Duration smoked	3.15 (2.42–4.11)	<0.001	2.03 (1.49–2.75)	<0.001
+Vit D, duration smoked	3.07 (2.35–4.01)	<0.001	2.04 (1.50–2.77)	<0.001
+Vit D, duration smoked, NSAID use	3.05 (2.33–3.98)	<0.001	2.03 (1.50–2.76)	<0.001
+Vit D, duration smoked, NSAID use, diabetes	3.05 (2.33–3.98)	<0.001	2.04 (1.50–2.77)	<0.001
+Vit D, duration smoked, NSAID use, BMI	3.05 (2.33–3.98)	<0.001	2.02 (1.48–2.74)	<0.001

Table 4

Association of factors related to inflammation with melanoma specific death, adjusted for age and sex

	Univariable			Multivariable (n=1570)	
Factor	**HR (95% CI)**	**p-value**	**n**	**HR (95% CI)**	**p-value**
Vitamin D per 10 nmol/L*	0.89 (0.83– 0.95)	<0.001	1600	0.90 (0.85– 0.96)	0.002
Vitamin D categorical*			1600		
<20nmol/l	2.19 (1.43–3.34)	<0.001			
20–60 nmol/l (baseline)	1.0	-			
60–85 nmol/l	0.86 (0.63–1.17)	0.4			
85–100 nmol/l	0.59 (0.24–1.44)	0.2			
100+ nmol/L	0.46 (0.11–1.85)	0.3			
BMI per 5 units	1.04 (0.91–1.18)	0.6	1935		
BMI			1935		
< 18.5	1.26 (0.40–3.96)	0.7		1.29 (0.41–4.09)	0.7
18.5–25 (baseline)	1.0	-		-	-
25–30	0.70 (0.53–0.93)	0.01		0.70 (0.52–0.95)	0.02
> 30	1.12 (0.83–1.50)	0.5		1.09 (0.79–1.51)	0.6
NSAIDs (ever/never) (BNF 10.1.1)	0.74 (0.49–1.12)	0.2			
Duration of aspirin use	0.98 (0.94–1.03)	0.4	1931		
Diabetes	1.55 (0.96–2.52)	0.07	1871		
Smoking					
Ever smoked (yes/no)	1.35 (1.06–1.71)	0.01	1884		
Still smoke (yes/no)	1.64 (1.17–2.30)	0.004	1885		
Duration (per 10 years)	1.13 (1.05–1.22)	0.001	1876	1.15 (1.07–1.24)	<0.001
Pack years (inc. rolls and cigars per 10 packyears)	1.00 (0.97–1.02)	0.8	1865		

* Vitamin D adjusted for season and batch. Given values assume that blood was drawn in spring and sampled from the first batch.

Table 5

Association of factors related to inflammation with melanoma specific death, adjusted for age, sex, site of primary and Breslow thickness

	Univariable			Multivariable (n=1557)	
Factor	**HR (95% CI)**	**p-value**	**n**	**HR (95% CI)**	**p-value**
Vitamin D per 10 nmol/L*	0.94 (0.88–1.00)	0.05	1585		
Vitamin D categorical*			1585		
<20nmol/l	1.79 (1.15–2.78)	0.009		1.52 (0.97–2.40)	0.07
20–60 nmol/l (baseline)	-	-		-	-
60–85 nmol/l	0.91 (0.66–1.24)	0.5		0.92 (0.66–1.27)	0.6
85–100 nmol/l	0.58 (0.24–1.42)	0.2		0.62 (0.25–1.52)	0.3
100+ nmol/l	0.67 (0.17–2.70)	0.6		0.67 (0.17–2.73)	0.6
BMI per 5 units	0.91 (0.80–1.03)	0.2	1915		
BMI			1915		
< 18.5	0.89 (0.22–3.62)	0.9		0.95 (0.23–3.88)	0.9
18.5–25 (baseline)	-	-		-	-
25–30	0.73 (0.55–0.97)	0.03		0.72 (0.53–0.98)	0.04
30+	0.95 (0.70–1.29)	0.7		0.95 (0.68–1.33)	0.8
NSAIDs (ever/never) (BNF 10.1.1)	0.80 (0.52–1.23)	0.3	1929		
Duration of aspirin use	0.99 (0.94–1.04)	0.6	1911		
Diabetes	1.31 (0.80–2.12)	0.3	1855		
Smoking					
Ever smoked (yes/no)	1.24 (0.97–1.57)	0.09	1867		
Still smoke (yes/no)	1.38 (0.97–1.95)	0.07	1868		
Duration (per 10 years)	1.10 (1.02–1.18)	0.01	1859	1.11 (1.03–1.20)	0.009
Pack years (inc. rolls and cigars per 10 packyears)	1.00 (0.97–1.03)	0.8	1848		

* Vitamin D adjusted for season and batch. Given values assume that blood was drawn in spring and sampled from the first batch.