Corso di dottorato di ricerca in:

"Alimenti e Salute Umana"

Ciclo 32°

Titolo della tesi

"Analisi dello status ossidativo in pazienti con melanoma cutaneo"

Dottorando
Dott.ssa Cinzia Buligan

Supervisore
Chiar.mo Prof. Giuseppe Damante

Co-supervisori
Chiar.mo Prof. Giuseppe Stinco
Chiar.ma Prof.ssa Sabina Cauci

Anno 2020

Alla mia famiglia:

le mie radici ed i miei rami.

INDICE

1. SINTESI

La presente tesi di Dottorato rappresenta uno dei rami di un progetto di ricerca più ampio incentrato sul melanoma cutaneo che è stato portato avanti durante i tre anni del Corso di Dottorato dalla Clinica Dermatologica dell'Azienda Sanitaria Universitaria di Udine, diretta dal prof. Stinco, e dal Laboratorio di Biochimica Clinica e Biologia Molecolare Clinica afferente al Dipartimento di Area Medica dell'Università di Udine di cui la prof.ssa Cauci è responsabile. Il lavoro sinergico di queste strutture ha portato finora alla pubblicazione di tre lavori che rappresentano altrettante ramificazioni del percorso di cui questa tesi fa parte. Essendo parte integrante del lavoro svolto durante il Corso di Dottorato, si allegano in Appendice le pubblicazioni e se ne riassumono di seguito i principali risultati.

Il primo lavoro, intitolato "BsmI (rs1544410) and FokI (rs2228570) vitamin D receptor polymorphisms, smoking, and body mass index as risk factors of cutaneous malignant melanoma in northeast Italy" (Cauci et al. 2017), ha indagato il ruolo di due polimorfismi del recettore della vitamina D (VDR), BsmI e FokI, nel melanoma cutaneo. Come in tutti i lavori, la popolazione oggetto di studio era esclusivamente appartenente a residenti in Friuli Venezia-Giulia, al fine di ottenere un background etnico omogeneo. Sono stati analizzati i polimorfismi del VDR mediante la tecnica di analisi della lunghezza dei frammenti di restrizione in 120 pazienti con melanoma (68 non metastatici e 52 metastatici) e 120 controlli sani. L'assenza o la presenza del sito di restrizione di BsmI e FokI è stata indicata rispettivamente con B" e "F" o con "b" e "f". Dai risultati è emerso come il genotipo bb di BsmI fosse più frequente tra i melanomi metastatici rispetto ai non metastatici (OR=3.18). Confrontando i pazienti con melanoma e i soggetti sani sono emersi i seguenti biomarcatori di maggior rischio: storia di fumo di sigaretta ≥20 anni isolata (OR=2.43) e combinata con genotipo bb (OR=4.78), Bb+bb (OR=2.30), Ff (OR=3.04), e Ff+ff (OR=3.08); obesità (BMI>30 kg/m2) isolata (OR=3.54) e combinata con Bb+bb (OR=3.52), Ff (OR=4.78), e Ff+ff (OR=6.56). Nel confronto tra melanomi metastatici e non metastatici sono emersi i seguenti biomarcatori di rischio: storia di fumo di sigaretta ≥20 anni isolata (OR=2.39) e combinata con bb (OR=5.13), Bb+bb (OR=3.07), e Ff+ff (OR=2.66); obesità combinata con Bb+bb (OR=5.27), Ff (OR=6.28), e Ff+ff (OR=9.18). La triplice presenza di fumo ≥20 anni, obesità e Bb+bb aveva un OR=9.18 per i melanomi rispetto ai sani e un OR=12.2 per metastatici rispetto ai non metastatici. Pertanto, il lavoro ha evidenziato come i polimorfismi di VDR associati a durata del fumo e obesità rappresentino possibili fattori di rischio per il melanoma (Cauci et al. 2017).

Nella seconda pubblicazione, intitolata "Immunohistochemical evaluation of vitamin D receptor (VDR) expression in cutaneous melanoma tissues and four VDR gene polymorphisms" (La Marra et al. 2017), sono stati aggiunti dati relativi anche all'espressione del VDR a livello tissutale, attraverso la tecnica di immunocolorazione, e l'analisi molecolare di quattro polimorfismi di VDR: BsmI, FokI, ApaI e TaqI, la cui assenza o presenza del sito di restrizione è stata connotata dalla lettera maiuscola o minuscola per ciascun polimorfismo. La popolazione oggetto di studio comprendeva 74 pazienti con melanoma (51.4% in stadio I, 21.6% in stadio II, 13.5% in stadio III, e 13.5% in stadio IV). L'espressione di VDR tissutale è stata classificata come: 100% positivo vs. <100%; oltre il 20% della mediana (alta espressione di VDR) vs. ≤20% (bassa espressione di VDR); assenza vs. presenza di cellule che esprimono VDR. DA i risultati è emerso come l'elevata espressione di VDR rispetto a quella bassa era più frequente nei melanomi in stadio I, con spessore di Breslow<1.00mm, con livello II di Clark, genotipo eterozigote Aa e genotipo combinato AaTT. Invece i genotipi combinati BbAA, bbAa, AATt, BbAATt e bbAaTT erano più frequenti nelle cellule che esprimevano 100% VDR vs. <100%; invece il genotipo AATT era più frequente nei melanomi che non esprimevano VDR (OR=14.5, P=0.025). L'espressione di VDR non era associata con metastasi, ulcerazione, mitosi>1, regressione, infiltrato linfocitario tumorale, emboli vascolari, familiarità per melanoma e presenza di altri tumori cutanei e non. Pertanto, il lavoro ha evidenziato

nell'insieme un'influenza dei polimorfismi del VDR sull'espressione tissutale di VDR stesso nel melanoma, con possibili implicazioni relative alla supplementazione di vitamina D nel melanoma.
Nell'ultima pubblicazione dal titolo "Interleukin 1 receptor antagonist gene variable number of tandem repeats polymorphism and cutaneous melanoma" (Cauci et al. 2019) è stato invece studiato il ruolo del polimorfismo del gene dell'antagonista del recettore dell'IL-1 nel melanoma, confrontando 133 soggetti con melanoma (72 con melanoma non metastatico e 61 metastatici) e 382 controlli sani. Sulla base del numero di ripetizioni di 86 paia di basi, sono stati identificati 5 alleli di IL-1RN: allele 1 (4 ripetizioni), allele 2 (2 ripetizioni – allele corto), allele 3 (5 ripetizioni), allele 4 (3 ripetizioni), allele 5 (6 ripetizioni). Gli alleli con 3 o più ripetizioni (es. allele 1, 3, 4 e 5) sono stati definiti ripetizioni lunghe e nominati con L. Le analisi dei dati hanno dimostrato come i genotipi 1/2 e 2/L fossero più frequenti tra i pazienti con melanoma rispetto ai sani (rispettivamente OR=1.84, P=0.003 e OR=1.66, P=0.002); al contrario il genotipo 1/1 risultava meno frequente tra i melanomi rispetto ai controlli (OR=0.62, P=0.017). Non erano emerse differenze significative nel confronto tra metastatici e non metastatici. I risultati hanno dimostrato quindi come il polimorfismo di IL-1RN possa influire sulla suscettibilità alla malattia, in quanto alcuni genotipi risultavano protettivi e altri possibili fattori di rischio nei confronti del melanoma, introducendo tale polimorfismo tra i possibili biomarcatori per il melanoma (Cauci et al. 2019).
Successivamente, dalla partecipazione ad attività di ricerca coordinate dalla Prof. Cauci sullo stress ossidativo nelle atlete (Cauci S, Buligan C, Marangone M, Francescato MP. Oxidative stress in female athletes using combined oral contraceptives. Sports Med Open. 2016;2(1):40), è nato il progetto della presente tesi, il cui focus si è spostato sul ruolo dello stress ossidativo nel melanoma. Sono stati quindi esaminati 240 pazienti in totale, di cui 120 pazienti con melanoma (53 con malattia metastatica e 67 con malattia non metastatica) e 120 soggetti sani. Per ciascun paziente sono stati calcolati i seguenti parametri di stress ossidativo: il livello di idroperossidi sierici e la quota di capacità antiossidante totale, utilizzando i test FORT e FORD (Callegari, Italia), rispettivamente. I risultati ottenuti hanno evidenziato come i pazienti con melanoma abbiano livelli di difese antiossidanti (FORD) inferiori rispetto ai controlli sani ($P<0.001$); inoltre i pazienti con melanoma metastatico presentavano livelli di perossidazione lipidica (FORT) circa 3 volte superiori ai pazienti non metastatici ($P=0.001$) e ai controlli sani ($P=0.001$) e valori di capacità antiossidante molto inferiori ai soggetti sani ($P<0.001$). Riguardo al complesso tema dello stress ossidativo e melanoma, sono pochi gli studi in Letteratura eseguiti su pazienti ed il presente lavoro contribuisce ad arricchire la Letteratura in tal senso, richiamando l'attenzione sul ruolo dello stress ossidativo soprattutto nel rischio di metastasi.
Tutti i lavori sono stati condotti considerando la validità dell'approccio diagnostico/terapeutico della medicina di precisione, che pone l'attenzione sulla variabilità individuale riguardo ad aspetti genetici, ambientali e stili di vita.
Pertanto, con il supporto dei precedenti lavori che suggerivano un'implicazione dell'interazione geni-ambiente nella suscettibilità e severità del melanoma e della recente Letteratura che evidenzia un ruolo anti-carcinogenetico della vitamina D nel melanoma attraverso l'inibizione dello stress ossidativo (Philips et al. 2020), l'obiettivo futuro sarà quello di analizzare globalmente i dati relativi ai polimorfismi del VDR e quelli dello stress ossidativo per ottenere un quadro il più possibile generale e comprensivo della complessa biologia del melanoma cutaneo.

2. IL MELANOMA: EPIDEMIOLOGIA E STRATEGIE DI PREVENZIONE

I dati epidemiologici degli ultimi anni mostrano per il melanoma cutaneo un'incidenza in continuo aumento, con differenze geografiche e socioeconomiche riguardanti anche lo stadio alla diagnosi e l'outcome della malattia. A fronte di dati che indicano un numero di casi sempre crescente, si rende sempre più necessario attuare adeguate misure preventive che tengano anche conto delle differenze sopra riportate (Strömberg et al. 2016; Guy et al. 2015). Le stime di crescita a livello mondiale indicano come nei prossimi 20 anni ci sarà un aumento del numero di casi mondiale in entrambi i sessi del +62,3%. Questo dato interesserà presumibilmente soprattutto le popolazioni di Nord-America, Europa e Australia, dove, secondo i dati del Global Cancer Observatory, insorgono circa l'85% dei melanomi cutanei (http://globocan.iarc.fr). In Europa non esistono in tutti gli stati registri tumorali centralizzati di alta qualità per cui gli studi riportano importanti discrepanze tra i paesi del centro e del nord Europa rispetto a quelli del Sud e dell'Est europeo. I dati EUCAN variano ampiamente da 50.3 casi per 100 mila abitanti in Norvegia ai 5.7 casi per 100 mila abitanti in Romania (Figura 1). Riguardo al sesso l'incidenza per il sesso maschile è di 19.8 casi/100.000 abitanti, mentre per le donne di 19 (https://ecis.jrc.ec.europa.eu/).

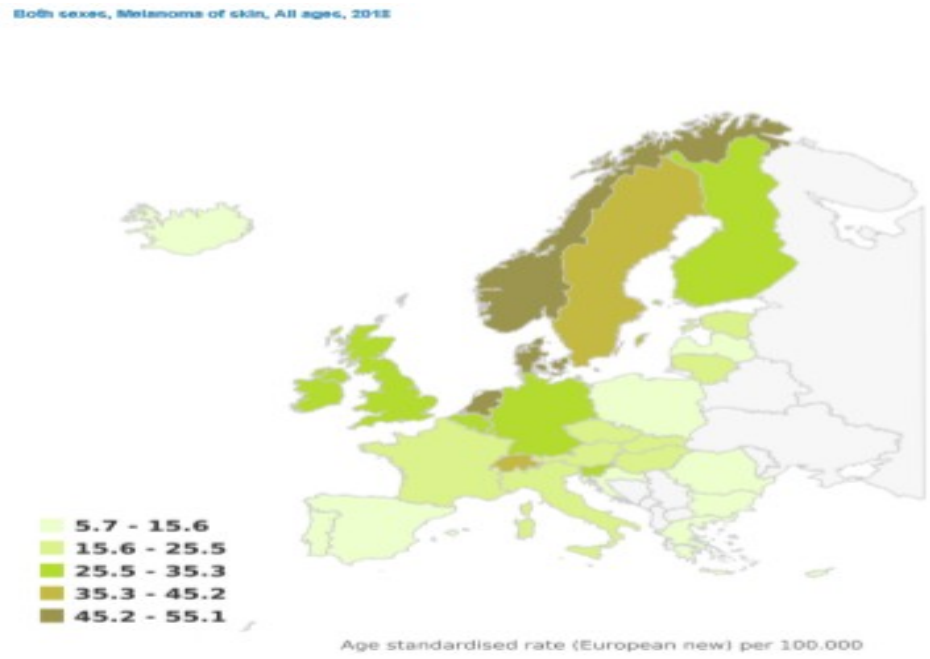
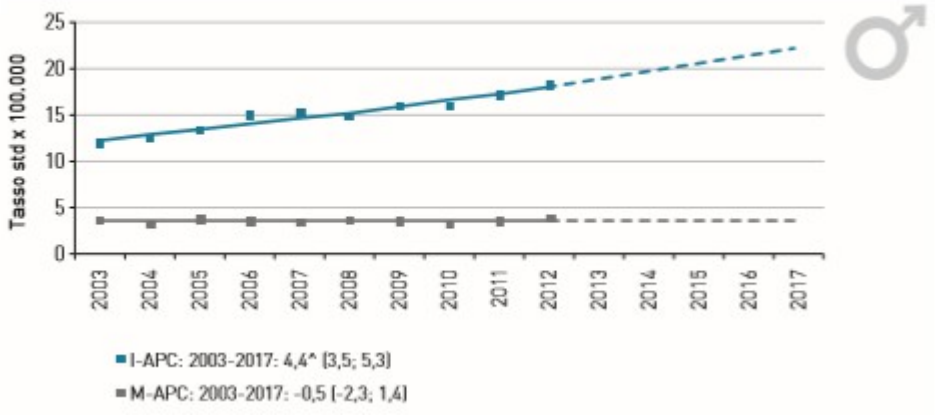


Figura 1. Tasso di incidenza standardizzato per età per melanoma maligno della cute

Dati registro europeo EUCAN 2018

Guardando al panorama nazionale italiano, secondo i database, l'incidenza del melanoma in Italia è in continuo aumento; si calcola che sia il secondo tumore più frequente negli uomini e il terzo nelle donne al di sotto dei 50 anni. Gli ultimi dati acquisiti mostrano un'incidenza standardizzata per età del 22.5 nei maschi e del 16/100mila abitanti nelle femmine (https://ecis.jrc.ec.europa.eu/). I trend di incidenza dal 2003 al 2017 espressi come APC (Annual Percent Change) mostrano un alto incremento sia per gli uomini che per le donne (+4.4% e +3.0% per anno), a fronte di un tasso di mortalità in diminuzione (AIOM-AIRTUM et al. 2017) (Figura 2). Come avviene per molti tumori, anche per il melanoma, esiste un gradiente Nord-Sud per i principali indicatori tumorali. In particolare, per il melanoma si confermano tassi d'incidenza doppi al Nord rispetto al Sud/Isole per entrambi i sessi. Alla base di queste differenze possono essere chiamati in causa diversità negli stili di vita e minore esposizione a fattori cancerogeni, riferiti anche a molti anni prima. Tuttavia, è possibile che il fenomeno dell'omogeneizzazione degli stili di vita che si sta attuando nel nostro paese porterà nel tempo a livellare queste differenze (AIOM-AIRTUM et al. 2019).

(A)



(B)

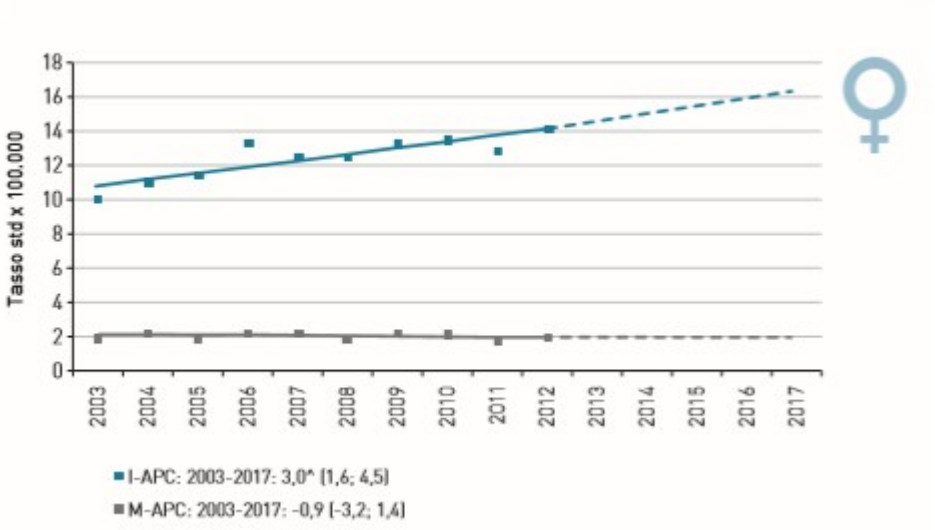


Figura 2. (A) Maschi (B) Femmine. Stima dei trend tumorali di incidenza e mortalità 2003-2017 per il melanoma. Tassi standardizzati nuova popolazione europea 2013. APC = Annual Percent Change (variazione percentuale media annua), I = incidenza, M = mortalità.

Al nord emerge inoltre come la più alta prevalenza si registri nelle regioni del nord-est, con un valore di 252/100.000 abitanti (www.tumori.net).

Nella regione Friuli Venezia-Giulia i dati stimati per il 2019 indicano un tasso di incidenza standardizzato per età di 30,5/100.000 abitanti per gli uomini e 23 per le donne. Sebbene il dato per il sesso femminile sia sovrapponibile a quello di altre regioni spicca invece il valore dei dati maschili, che è tra i più alti d'Italia. La prevalenza è di 254/100.000 abitanti (www.tumori.net). Inoltre, una recente analisi sull'incidenza del melanoma in FVG ha dimostrato un trend crescente già nel periodo 1995-2005, mostrando inoltre come l'area di Trieste e la zona costiera del FVG presentino tassi di incidenza standardizzati molto alti, attorno ai 20/100.000 abitanti in entrambi i sessi (Figura 3) (Cecconi et al. 2016).

ASS	Male				Female			
	Cases	Crude rate	Standardised rate*	SIR	Cases	Crude rate	Standardised rate*	SIR
ASS 1 (Triestina)	355	28.45	19.67	1.40	340	24.00	18.40	1.25
ASS 2 (Isontina)	175	24.23	18.44	1.28	160	20.53	15.52	1.11
ASS 3 (Alto Friuli)	44	10.84	8.36	0.59	68	15.80	11.09	0.88
ASS 4 (Medio Friuli)	288	16.24	12.74	0.88	321	16.62	12.54	0.93
ASS 5 (Bassa Friulana)	100	17.54	13.83	0.96	111	18.62	13.48	1.06
ASS 6 (Friuli Occidentale)	199	12.93	10.65	0.75	222	13.80	10.25	0.80
Total	1161	18.55	14.23	1.00	1222	18.07	13.51	1.00

ASS, *azienda per l'assistenza sanitaria* (healthcare assistance company); SIR, standardised incidence ratio. *Direct standardisation on European population.

Figura 3. Numero di casi, tasso di incidenza grezzo e standardizzato e SIR (standardized incidence ratio) per melanoma in Friuli Venezia Giulia (1995-2005), per sesso.

Secondo i dati sopraesposti, il numero di casi di melanoma è destinato a crescere nei prossimi anni e con esso un aumento dei costi per il sistema sanitario, se non verranno messe in atto valide strategie di prevenzione della malattia. Tra queste, di fondamentale importanza appaiono la riduzione dell'abitudine all'esposizione solare e alle lampade abbronzanti, la sensibilizzazione all'uso di schermi solari protettivi e la prevenzione delle scottature solari (Guy et al. 2015). Oltre a ciò, lo stile di vita può giocare un ruolo importante nel modulare il rischio di melanoma. In particolare,

l'alimentazione potrebbe rivelarsi utile al fine della prevenzione del melanoma. Infatti, molti agenti antiossidanti in fase di sperimentazione per la prevenzione del melanoma sono derivati alimentari (Cassidy et al. 2012). I dati però rimangono ancora contrastanti. Interessanti sono anche i dati relativi alla correlazione tra il consumo di caffè e la riduzione del rischio di melanoma (Micek et al. 2018). Un ruolo rilevante ancora da chiarire spetta all'esposizione ad agenti cancerogeni, quali il fumo di sigaretta (Stadler et al. 2019; Cauci et al. 2017) e gli inquinanti ambientali. Infine, rimane da approfondire l'azione della vitamina D sul rischio di melanoma (Cauci et al. 2017; La Marra et al. 2017)

3. LO STRESS OSSIDATIVO

Il concetto di stress ossidativo è stato introdotto nel 1985 da Sies H. in un capitolo introduttivo del libro intitolato "Oxidative Stress" (Sies H. 1985). Da allora, la biologia dei processi ossido-riduttivi è diventata un'area di ricerca in continuo sviluppo in diversi ambiti, dalla biochimica alla fisiologia cellulare fino ad arrivare alla biologia generale e alla medicina. Successivamente il concetto di stress ossidativo è stato esteso, includendo i segnali che regolano i processi ossidazione-riduzione, con lo scopo di ridefinire lo stress ossidativo stesso (Jones et al. 2006; Jones et al. 2008; Lichtenberg et al. 2015).

Fisiologicamente l'ossidazione è un processo che avviene quando l'ossigeno si combina con molecole ridotte, come i carboidrati, i lipidi o le proteine per produrre energia. Nello stesso processo, si producono però prodotti intermedi noti come radicali liberi, alcuni dei quali possono iniziare altri processi ossidativi determinando una reazione a catena. Pertanto, gran parte dei processi biologici generano normalmente radicali liberi che possono avere effetti addirittura benefici (ad es., i processi di difesa immunitaria), ma quando la formazione dei radicali liberi diventa eccessiva, essi possono essere estremamente distruttivi e attaccare componenti fondamentali delle cellule come lipidi, proteine e DNA (Halliwell et al. 2012; Palmieri et al. 2007). I radicali liberi sono di solito inattivati o rimossi da un sistema naturale di difesa antiossidante (in parte endogeno, in parte introdotto con gli alimenti) con lo scopo di prevenire il danno cellulare indotto dai radicali stessi. In generale, gli antiossidanti possono essere suddivisi in:

· meccanismi enzimatici (ad es. superossido dismutasi, catalasi e glutatione perossidasi);

· meccanismi non enzimatici (vitamina E, vitamina C, carotenoidi, tioli, flavonoidi, albumina, glutatione, metalloproteine,ecc.).

In condizioni normali, le naturali capacità di difesa dell'organismo sono in grado di neutralizzare totalmente l'azione dei radicali liberi, ma se si formano radicali liberi in eccesso e/o tali sostanze ad azione antiossidante sono poche o inefficaci, si va incontro al danno ossidativo e si instaura uno stato di stress ossidativo cioè una condizione di sbilanciamento tra sostanze ossidanti ed antiossidanti (Cannavò et al. 2019; Sies et al. 2017; Sanders et al. 2004; Kadekaro et al. 2012). (Figura 4)

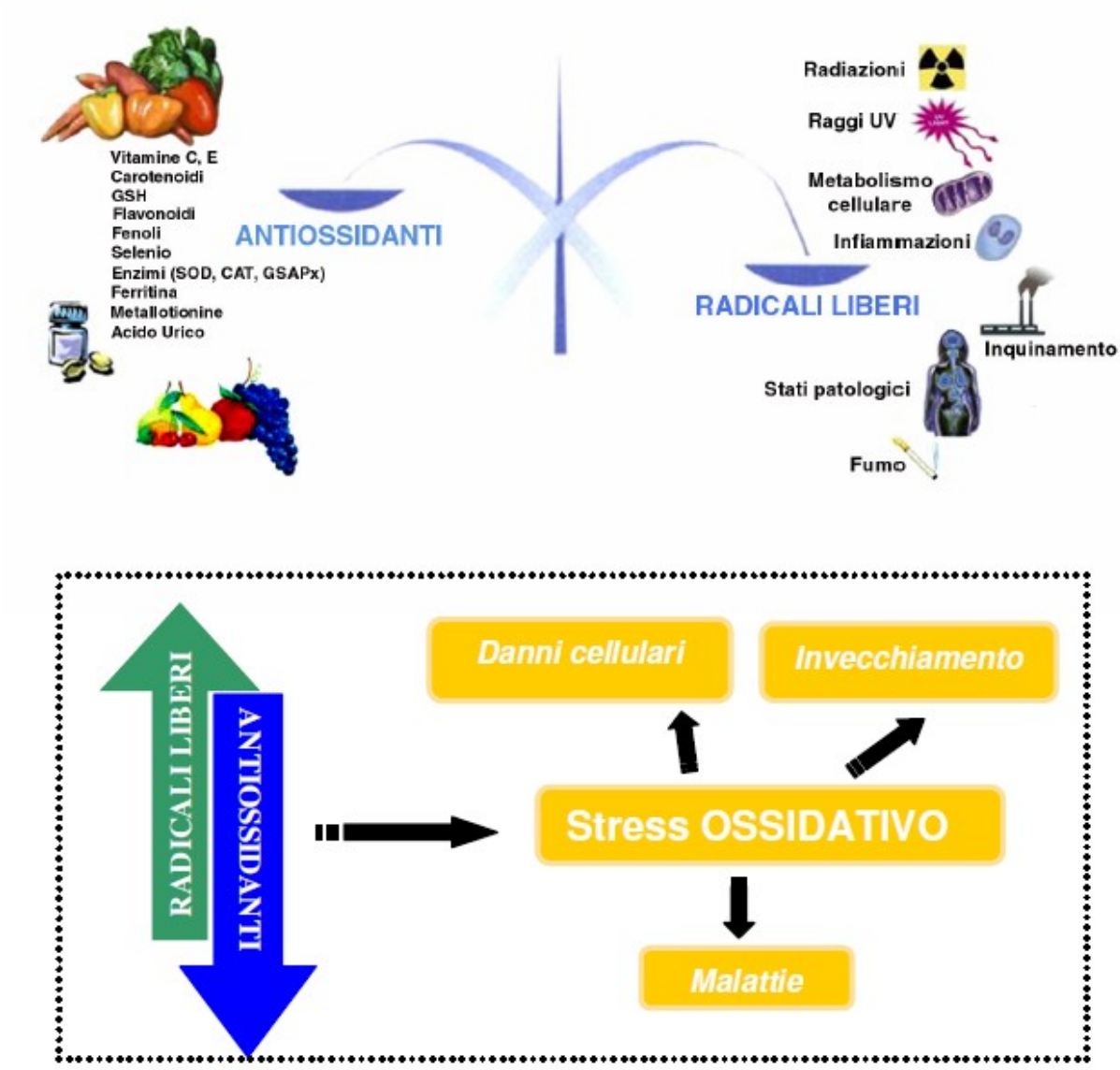


Figura. 4. Genesi dello stress ossidativo

In Letteratura, ci sono sempre più evidenze di un coinvolgimento dello stress ossidativo nella patogenesi di diverse condizioni patologiche, tra cui malattie cardiovascolari, neurologiche, stati tumorali, infiammazioni (Carvalho et al. 2017; Crotty et al. 2017; Sanders et al. 2004; Pignatelli et al. 2018).

Inoltre, data l'enorme varietà di molecole antiossidanti e pro-ossidanti, lo sforzo scientifico è volto a classificare diverse sottoforme di stress ossidativo e ad introdurre delle scale di intensità variabili dallo stress ossidativo fisiologico fino al carico ossidativo eccessivo o tossico (Sies H. 2015).

4. MISURAZIONE DELLO STRESS OSSIDATIVO

Negli ultimi anni sono stati studiati e prodotti diversi test di laboratorio per la valutazione della capacità antiossidante totale plasmatica o sierica e degli indici di danno ossidativo. La combinazione di biomarcatori di stress ossidativo permette una valutazione globale del bilancio redox dell'organismo, dei bisogni nutrizionali e delle eventuali strategie antiossidanti, oltre che fornire indicazioni sul potenziale ruolo dello stress ossidativo nella patogenesi di molte condizioni morbose (Palmieri 2007).

4.1 TEST FORT

Il FORT (Free Oxygen Radicals Test) è un test colorimetrico basato sulla capacità dei metalli di transizione (es., il ferro) di catalizzare la scissione degli ROOH (idroperossidi, specie radicaliche) presenti nel campione biologico in derivati radicalici secondari, secondo la classica reazione di Fenton. Dopo che si sono formati a livello cellulare, gli ROOH mantengono la propria reattività chimica e sono in grado di generare quantità proporzionali di prodotti alcossilici, $RO^{\cdot}$, e perossilici, $ROO^{\cdot}$ (reazioni 1 e 2). Queste molecole vengono, poi, intrappolati da uno specifico derivato amminico (reagente R1 del FORT test, CrNH2) sviluppando, in una reazione cinetica lineare a 37°C, un catione radicalico (Cr-NH2 $^{+\cdot}$, reazione 3) più stabile nel tempo e colorato perciò misurabile fotometricamente. Pertanto, secondo la legge di Lambert-Beer, l'intensità del colore sviluppatosi durante la reazione correla direttamente con la quantità dei derivati radicalici e, di conseguenza, con lo stato ossidativo del campione analizzato.
Le reazioni coinvolte nel FORT test sono:

1. $R\text{-}OOH + Fe^{2+} \rightarrow R\text{-}O^{\bullet} + OH^{-} + Fe^{3+}$

2. $R\text{-}OOH + Fe^{3+} \rightarrow R\text{-}OO^{\bullet} + H^{+} + Fe^{2+}$

3. $RO^{\bullet} + ROO^{\bullet} + 2CrNH_2 \rightarrow RO^{-} + ROO^{-} + [Cr\text{-}NH_2^{+\bullet}]$

Data l'eterogeneità chimica delle specie reattive secondarie derivanti dalla scissione ferro-dipendente degli ROOH nella reazione del FORT test, è stato deciso, per semplicità d'interpretazione, di convertire i valori di assorbanza misurati in unità convenzionali chiamate unità FORT. La trasformazione viene eseguita automaticamente dallo strumento così che i risultati sono immediatamente valutabili dall'operatore e di più facile interpretazione.
Per consentire una valutazione assoluta, i risultati possono essere espressi anche in concentrazioni equivalenti di H2O2 usato quale idroperossido di riferimento. Una unità FORT corrisponde a circa 0,25 mg/l – 7,5 mmol/l di H2O2.
I fotometri della serie CR3000/FORM sono calibrati con una curva di riferimento di diverse concentrazioni di H2O2 memorizzata nel microprocessore in modo tale che i valori di assorbanza vengano automaticamente convertiti in unità FORT e/o concentrazioni corrispondenti di H2O2.
Intervallo di linearità: 160-600 unità FORT.
Valori di riferimento: sono considerati normali valori sino a circa 310 unità FORT corrispondenti a 2,35 mmol/l H2O2 (Harma et al. 2006; Palmieri et al. 2006).
L'intervallo di normalità del FORT deriva da anni di raccolta diretta dei dati, oltre ad essere stato confermato da uno studio di popolazione (Dal Negro et al. 2003).

4.2 TEST FORD

Il FORD (Free Oxygen Radicals Defence) test determina la capacità antiossidante totale nel plasma. L'organismo umano possiede numerosi sistemi di difesa ad azione antiossidante fondamentali per prevenire l'eccessivo innalzamento del livello di radicali liberi e, quindi, mantenere sotto controllo lo stato ossidativo. Questi antiossidanti sono in parte fisiologici, tra cui acido urico, bilirubina, ceruloplasmina, transferrina, tioli, glutatione, ecc., e in parte derivano dagli alimenti (soprattutto frutta e verdura) tra cui le vitamine E, C, ed A, polifenoli, flavonoidi, carotenoidi ect. Considerate sia la complessità e l'interdipendenza dei vari sistemi antiossidanti e la loro indispensabile funzione per limitare i danni da radicali liberi, sia l'importante influenza di abitudini, dieta, supplementazioni e stile di vita nel determinare lo stato ossidativo globale di un individuo, la possibilità di valutare le difese antiossidanti disponibili risulta di primaria rilevanza.
Il FORD test è un test colorimetrico basato sulla formazione di un radicale colorato che si riduce in presenza di sostanze ad azione antiossidante. In ambiente acido (pH=5,2), un opportuno ossidante (FeCl3) reagisce con il cromogeno non colorato (Cromogeno, reagente FORD C1) formando il

corrispondente catione radicalico (Cromogeno·+): in questa forma il cromogeno è stabile e colorato; è perciò possibile effettuare misure fotometriche a 505nm (reazione 1). I composti antiossidanti (AOH) presenti nel campione aggiunto riducono il catione radicalico determinando una scomparsa del colore della soluzione proporzionale alla loro quantità (reazione 2).

I valori di assorbanza letti vengono trasformati in concentrazioni equivalenti applicando la legge di Lambert Beer, facendo riferimento alla curva standard ottenuta con Trolox (6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), un derivato della vitamina E permeabile alle cellule con elevate proprietà antiossidanti e perciò comunemente usato come standard.

Reazioni coinvolte nel FORD test:

1. Cromogeno $_{(incolore)}$ + Fe^{2+} + H^{+} $\rightarrow$ Cromogeno$^{\bullet+}{}_{(porpora)}$

2. Cromogeno$^{\bullet+}{}_{(porpora)}$ + AOH $\rightarrow$ Cromogeno$^{+}{}_{(incolore)}$ + AO

Intervallo di linearità: 0.25-3.0 mmol/l Trolox.

Valori di riferimento: 1.07 – 1.53 mmol/l Trolox equivalenti. (Palmieri et al. 2006; Torri et al. 2006)

Sulla base dei valori di riferimento riportati in letteratura, al momento i valori di normalità del test FORD sono stimati essere inclusi nell'intervallo 1.07-1.53 mmol/l Trolox eq. (valore medio = 1.23 mmol/l Trolox eq) che comprende circa l'85% dei valori (Yeum et al. 2004).

5. STUDIO SPERIMENTALE

5.1 INTRODUZIONE

Il 60-70% dei melanoma cutanei originano dall'esposizione ai raggi ultravioletti (UV) (Koh et al. 1996). La radiazione UV di origine solare o per esposizione a lettini abbronzanti determina un danno al DNA UV-indotto, stress ossidativo e flogosi cutanea (Sample and He. 2018). In particolare, il danno al DNA può avvenire per meccanismi diretti o mediati. Tra le reazioni dirette all'esposizione ai raggi UVB (280 – 315 nm) sono descritti la formazione di dimeri di pirimidina ciclobutano ad azione mutagenica, fotoprodotti, rotture della doppia elica e doppi legami. Se il danno non viene correttamente riparato può generare mutazioni geniche che contribuiscono alla carcinogenesi cutanea. Dall'altra parte, l'azione dei raggi UVA (315-400nm), che rappresentano il 95% della radiazione solare, è meno genotossica degli UVB, ma agisce comunque con un danno indiretto mediato dallo stress ossidativo, causando la formazione di radicali liberi dell'ossigeno (Kim and He. 2014). Nel melanoma, sembra che le mutazioni da riparo incorretto di fotoprodotti siano rare, suggerendo così un ruolo più importante del danno ossidativo nella trasformazione maligna dei melanociti (Song et al. 2009). Pertanto, un meccanismo chiave attraverso cui la radiazione UV provoca l'insorgenza del melanoma è la produzione di specie reattive dell'ossigeno (ROS) che includono anione superossido ($O_2^{\bullet-}$), radicale ossidrile ($HO^{\bullet}$) e perossido di idrogeno (H_2O_2) (Sample and He. 2018).

Normalmente i ROS sono prodotti nei mitocondri durante le reazioni metaboliche ossigeno-dipendenti (Porporato et al. 2014). Inoltre, altre sorgenti endogene di ROS sono rappresentate dalla melenogenesi, dalle attività del citocromo P450, NADPH ossidasi, lipossigenasi, perossisomi e dall'infiammazione (Cannavò et al. 2019; Sies et al. 2017). Tra le sorgenti esogene di ROS, oltre alla radiazione UV, sono presenti radiazioni ionizzanti, tossine ambientali, prodotti chimici e metalli inquinanti come arsenico, cadmio e piombo (Denat et al. 2014; Zimta et al. 2019; Lopes et al. 2016).

Quando i ROS non sono adeguatamente neutralizzati dal pool di antiossidanti per iperproduzione di ROS o riduzione della capacità antiossidante si crea uno stato di stress ossidativo (Sies et al. 2017).

Lo stress ossidativo può distruggere l'omeostasi dei melanociti determinando la loro trasformazione maligna (Denat et al. 2014; Venza et al. 2015). E'stato dimostrato come livelli moderati di stress ossidativo supportino la proliferazione e la migrazione cellulare e attivino segnali indotti dallo stress coinvolti nella sopravvivenza cellulare, contribuendo quindi allo sviluppo del tumore (Denat

et al. 2014; Cannavò et al. 2019). Inoltre, lo stress ossidativo sembra avere un ruolo non solo nella trasformazione maligna del tumore primitivo, ma anche nella progressione e metastatizzazione (Venza et al. 2015; Obrador et al. 2018). Se il coinvolgimento dello stress ossidativo nella fase metastatica è stato maggiormente approfondito, recentemente è stato anche dimostrato come alterazioni molecolari che coinvolgono processi di stress ossidativo abbiano un ruolo nella fase di trasformazione del melanoma da invasione orizzontale a crescita verticale e quindi nella progressione precoce del tumore (Salhi et al. 2020). A supporto dell'associazione melanoma-stress ossidativo un recentissimo studio riporta un maggior rischio di melanoma nei soggetti esposti ad erbicidi; tra i meccanismi chiamati in causa si annoverano lo stress ossidativo oltre che il danno al DNA, l'infiammazione cronica e l'alterazione della risposta immunitaria (Stanganelli et al. 2020).
Dall'altra parte, in contrasto con l'ipotesi di associazione tra aumento dello stress ossidativo e carcinogenesi, in alcuni studi sperimentali, l'eccessivo stress ossidativo sembra compromettere la sopravvivenza dei melanociti determinandone la morte cellulare (Cannavò et al. 2019; Denat et al. 2014; Venza et al. 2015; Fischer et al. 2018).
Anche il ruolo degli antiossidanti usati in senso anti-tumorogenico rimane ancora dubbio. Infatti, in alcuni lavori, gli antiossidanti introdotti con la dieta sembrano prevenire il danno al DNA UV-indotto e lo stress ossidativo, oltre al fatto che alcuni studi di laboratorio hanno dimostrato che un'elevato consumo di antiossidanti inibisce lo sviluppo del melanoma e la progressione metastatica (Denat et al. 2014; Porporato et al. 2014; Goh et al. 2011). Molto di recente è stato anche dimostrato un ruolo benefico della vitamina D3 nel contrastare lo stress ossidativo e l'espressione di molecole infiammatorie e angiogenetiche in cellule di melanoma, esplicando in tal senso un ruolo anti-carcinogenetico (Philips et al. 2020). L'evidenza epidemiologica rimane tuttavia ancora incosistente. Infatti, in contrasto con quanto appena descritto, alcuni studi clinici prospettici su larga scala hanno mostrato un aumento dell' incidenza di tumore con l'assunzione nella dieta di antiossidanti (Klein et al. 2011, Omenn et al. 1996) e anche per il melanoma esistono prove in tal senso con l'assunzione di N-acetil-cisteina (Le Gal et al. 2015; Piskounova et al. 2015). Anche una recente metanalisi non supporta nessuna forte e significativa associazione tra melanoma e consumo di antiossidanti, nè di frutta e verdura (Miura et al. 2015).
Il tema della relazione tra melanoma e stress ossidativo è quanto mai dibattuto anche per il fatto che in Letteratura ci sono molti lavori che hanno riscontrato alti livelli di ROS nelle cellule di melanoma *in vitro* o in modelli animali (Picardo et al. 1996; Cannavò et al. 2019; Denat et al. 2014; Sander et al. 2004; Liu et al. 2012), ma ci sono pochi studi che hanno esaminato biomarcatori di stress ossidativo *in vivo* nei pazienti con melanoma (Gadjeva et al. 2008; Santos Bernardes et al. 2015; Santos Bernardes et al. 2016; Bisevac et al. 2018). Inoltre, i dati sulle cellule tumorali in coltura spesso non corrispondono a ciò che viene riscontrato nei pazienti (Obrador et al. 2018). Ad

oggi, i diversi pathways dello stress ossidativo non sono del tutto compresi e il ruolo dei ROS e degli antiossidanti nel melanoma rimane dubbio data la capacità contesto-dipendente di promuovere o inibire la tumorogenesi e le metastasi (Cannavò et al. 2019). Rimane pertanto da approfondire se lo stress ossidativo sia sistematicamente alto nei pazienti con melanoma e, in caso affermativo, se sia associato allo stadio del melanoma.
Il miglioramento delle conoscenze dei meccanismi che inducono stress ossidativo nei pazienti con melanoma potrebbe portare ad importanti risvolti nella complessa fisiopatologia dei danni indotti da stress ossidativo e potrebbe aprire la strada a nuovi studi anche sul ruolo degli antiossidanti nei pazienti con tumori cutanei.

5.2 OBIETTIVO DELLO STUDIO

L'obiettivo principale dello studio è quello di chiarire se i ROS e il sistema delle difese antiossidanti giochino un ruolo nello sviluppo del melanoma cutaneo e nella sua progressione, esaminando un numero di pazienti con melanoma e soggetti sani superiore a quello di precedenti studi su umani. A tale scopo, sono stati misurati i livelli di specie reattive dell'ossigeno, nella forma di idroperossidi e i livelli di molecole scavengers, le quali orchestrano le difese antiossidanti. Sono stati confrontati quindi i dati tra pazienti con melanoma e soggetti sani e tra pazienti con melanoma metastatico e pazienti con melanoma non metastatico.

5.3 MATERIALI E METODI

POPOLAZIONE IN STUDIO

Il presente studio caso-controllo è stato approvato dal Comitato Etico dell'Azienda Ospedaliera di Udine. Tutti i pazienti hanno firmato un consenso informato prima di sottoporsi allo studio.
Sono stati arruolati 120 pazienti (età 31-87 anni) con documentata diagnosi di melanoma, sottoposti ad escissione chirurgica di melanoma da 6 mesi a 23 anni (media±DS, 4.0±3.7 anni) prima dell'ingresso nello studio. I controlli erano 120 (età 31-87 anni) volontari sani che sono stati appaiati per sesso, età, origine familiare e status socio-economico con i casi. Tutti i pazienti sono stati arruolati durante le visite di routine effettuate presso la Clinica Dermatologica dell'Azienda Sanitaria Universitaria Friuli Centrale di Udine da Ottobre 2013 a Dicembre 2018.

I criteri d'inclusione sia per i casi che per i controlli erano: essere residenti in Regione FVG; avere almeno 2 nonni nati in FVG (o in territorio italiano prima della seconda Guerra mondiale, ma attualmente in Slovenia o in Croazia, come ad esempio la penisola dell'Istria); non avere più di 1 nonno originario dell'Italia centrale o meridionale. Per i casi di melanoma sono stati inclusi solo pazienti con melanoma cutaneo e non mucosale. Tra i criteri di esclusione, data la comprovata associazione tra assunzione di pillola estroprogestinica e aumento dello stress ossidativo, sono state escluse donne che assumevano terapie ormonali, e anche donne in stato di gravidanza, sia per i casi che per i controlli. Inoltre, per i controlli sono stati esclusi i pazienti con i seguenti criteri: pazienti affetti o con storia di altri tumori; pazienti con familiarità di primo grado per melanoma; pregressi trapianti d'organo; patologie acute (ad. es. infettive) o croniche (ad es. di tipo autoimmune quali diabete mellito tipo 1, artrite reumatoide, lupus eritematoso sistemico, etc).

La diagnosi di melanoma è stata effettuata sulla base delle caratteristiche immunoistologiche di nevi sospetti dal punto di vista clinico-dermoscopico che sono stati escissi chirurgicamente. La classificazione in stadi del melanoma è stata eseguita sulla base dei criteri clinici, istologici e radiologici secondo la classificazione clinico-patologica dell'American Joint Committee on Cancer (AJCC) 7^{th} (Balch et al. 2009). Per pazienti con melanomi multipli, è stato preso in considerazione per le analisi dello studio il melanoma principale ovvero quello più grave, sulla base della valutazione istologica di classificazione di tumore primario (T) secondo lo spessore tumorale in accordo con il grading T della classificazione TNM (Balch et al. 2009).

Ad ognuno dei soggetti è stato somministrato da un medico un questionario che approfondisse i fattori di rischio comportamentali e la storia clinica del paziente. Le informazioni raccolte includevano: caratteristiche demografiche, mediche e la storia di melanoma nella famiglia (genitori, fratelli o sorelle), abitudine al fumo, abitudini alimentari, scottature solari, ecc… (come riportato in Appendice). I pazienti affetti da melanoma hanno compilato, inoltre, con il medico, un questionario contenente informazioni sulla propria anamnesi, storia clinica e terapeutica. Il fototipo è stato valutato da un dermatologo esperto secondo i criteri di Fitzpatrick (Fitzpatrick 1988). L'indice di massa corporea (BMI) è stato calcolato come peso (kg) diviso per il quadrato dell'altezza (m). Valori di BMI superiori a 30 sono sati considerati indicatori di obesità.

VALUTAZIONE DELLO STRESS OSSIDATIVO

Per ogni partecipante è stato raccolto un campione di sangue capillare (20 μL) al mattino (tra le ore 8 e le 10) da seduto e dopo 12 ore di digiuno. Il personale addetto alla raccolta e all'analisi dei campioni non era a conoscenza dei dati demografici e clinici.

I ROS, sottoforma di perossidi di lipidi, sono stati valutati attraverso l'uso del FORT test (Callegari, Parma, Italy), un test colorimetrico della durata di 6 minuti dasato sulla capacità dei metalli di transizione di catalizzare la rottura di idroperossidi in radicali liberi secondo la reazione di Fenton. I risultati sono stati espresso in unità FORT (1 unità FORT corrisponde a 0.26 mg/L H_2O_2). La variabilità intra- e inter-procedura dovevano essere <5%. I valori limite erano ≤160 e ≥600 unità FORT. Valori superiori a 310 unità FORT sono stati considerati indicativi di aumentata perossidazione lipidica (Kamhieh-Milz and Salama. 2014; Cauci et al. 2016).

Un ulteriore campione di sangue capillare periferico (50 µL) è stato raccolto per la determinazione della capacità antiossidante totale tramite il test FORD (Callegari, Parma, Italy) (Francescato et al. 2014). I valori del test FORD sono espressi come mmol/L di equivalenti Trolox (Lewis et al. 2016; Cauci et al. 2016). La variabilità intra- e inter-procedura dovevano essere <5%. I valori limite erano ≤0.25 e ≥3.00 unità FORD. Nessun campione ha mostrato valori al di fuori di questo intervallo di rilevamento. I valori considerati normali secondo la casa prodruttrice Callegari erano compresi nell'intervallo 1.07 – 1.53 unità FORD, pertanto il valore di 1.1. unità FORD è stato considerato come valore soglia di un'elevata capacità antiossidante.

Il coefficente di riproducibilità di entrambi i test FORT (3.9%) e FORD (3.7%) erano sovrapponibili ai valori di stress ossidativo misurati in laboratorio ed avevano una precisione analitica sufficiente da poter essere usati nella pratica clinica (Lewis et al. 2016; Knott et al. 2015; Seyedsadjadi et al. 2017).

5.4 ANALISI STATISTICA

I dati sono presentati come media e deviazione standard (±DS). Il test di Mann-Whitney è stato usato per il confronto tra variabili continue. La differenza delle frequenze tra gruppi statificati è stata detrminata mediante il stratyificati il test χ^2 di Pearson o il test esatto di Fisher. Per le variabili categoriche sono stati calcolati gli Odds Ratios (ORs) e l'intervallo di confidenza al 95% (IC). Per alpha allo 0.05, avevamo una potenza del 90% di evidenziare una differenza di stress ossidativo tra i casi di melanoma ed i pazienti sani. Valori di $P<0.05$ erano considerati statisticamente significativi, mentre valori di $P<0.10$ indicavano una tendency. Le analisi statistiche sono state eseguite utilizzando il programma Statistical Package for Social Sciences (SPSS for Windows, SPSS Inc., Chicago, IL, USA).

5.5 RISULTATI

Nella tabella 1 sono riassunti i dati relativi al confronto dei parametri di stress ossidativo tra i pazienti con melanoma e i soggetti sani. Dai dati emerge come valori più elevati di perossidazione lipidica (≥310 unità FORT) siano leggermente più frequenti tra i 120 pazienti con melanoma (38.3%) rispetto ai controlli sani (28.3%), nonostante non ci sia una significatività statistica (P=0.10). Di converso, valori relativi ad una bassa capacità di difesa antiossidante (<1.1 unità FORD) erano molto più frequenti tra i pazienti con melanoma (72.5%) rispetto ai soggetti sani (30.0%) (OR=6.15, P<0.001) (Tabella 1).

Tabella 1. Confronto dei valori di perossidi (unità FORT) e della capacità antiossidante totale (unità FORD) tra 120 pazienti con melanoma e 120 soggetti sani.

	Tutti i soggetti (n=240)	**Casi di Melanoma (n=120)**	**Controlli sani (n=120)**	**OR (95% IC), Melanomi vs. sani**	**P-value Melanomi vs. sani**
Biomarcatori					
Idroperossidi, Unità FORT, media ±DS	322.8±99.9	329.6±65.2	298.7±95.7	-	-
Idroperossidi alti, FORT ≥310 Unità	80 (33.3%)	46 (38.3%)	34 (28.3%)	1.57 (0.91-2.70)	0.100
Capacità antiossidante totale, Unità FORD, media ±DS	1.09±0.22	1.04±0.21	1.22±0.21	-	-
Bassa capacità antiossidante totale, FORD <1.1 Unità	123 (51.2%)	87 (72.5%)	36 (30.0%)	**6.15 (3.52-10.8)**	**<0.001**

Riguardo al confronto tra pazienti metastatici e non metastatici, come illustrato nella tabella 2, gli elevati valori di stress ossidativo (≥310 Unità FORT) erano più frequenti nei melanomi metastatici (54.7%) rispetto ai non metastatici (25.4%) con un OR=3.55 e P=0.001. Inoltre, livelli di FORD <1.1 unità erano più frequenti nei pazienti con metastasi (83.0%) che nei non metastatici (64.2%), OR=2.73, P=0.022. Nella tabella 2 vengono inoltre mostrati i risultati di confronto tra pazienti con melanoma metastatico e controlli sani e pazienti con melanoma non metastatico e controlli sani. Avere valori elevati di stress ossidativo era più frequente nei pazienti con melanoma metastatico

rispetto ai controlli sani (OR=3.06, P=0.001). Allo stesso modo, bassi valori di difese antiossidanti (valori FORD <1.1 Unità) erano più frequenti nei melanoma metastatici che nei soggetti sani (OR=11.4, P<0.001.). Non sono invece emerse differenze significative nel confronto tra pazienti con melanoma non metastatico e soggetti sani per quanto rigurda i valori del test FORT. Invece, bassi valori di FORD (<1.1 Unità) erano significativamente più frequenti nei pazienti con melanoma non metastatico che nei controlli sani (OR=4.18, P<0.001) (Tabella 2).

Tabella 2. Confronto dei valori di perossidi (unità FORT) e della capacità antiossidante totale (unità FORD) nei pazienti con melanoma metastatico (MetM) e melanoma non metastatico (NMetM) e soggetti sani 120 pazienti con melanoma e 120 soggetti sani.

	Pazienti MetM (n=53)	**Patienti NMetM (n=67)**	**OR (95% IC), P-value, MetM vs. NMetM**	**OR (95% IC), P-value, MetM vs. sani**	**OR (95% IC), P-value, NMetM vs. sani**
Biomarcatori					
Idroperossidi, Unità FORT, media ±DS	303.1±67.5	298.7±65.2	-	-	-
Idroperossidi alti, FORT ≥310 Unità	29 (54.7%)	17 (25.4%)	**3.55 (1.64-7.69), 0.001**	**3.06 (1.56-5.98), 0.001**	0.86 (0.44-1.69), 0.663
Capacità antiossidante totale, Unità FORD, media ±DS	1.03±0.22	1.04±0.21	-	-	-
Bassa capacità antiossidante totale, FORD <1.1 Unità	44 (83.0%)	43 (64.2%)	**2.73 (1.14-6.54), 0.022**	**11.4 (5.04-25.8), <0.001**	**4.18 (2.22-7.88), <0.001**

Nella tabella 3 sono rappresentati i dati relativi al confronto dei pazienti con melanoma con alto (FORT≥310 unità) e basso (FORT <310 unità) stress ossidativo. I risultati statisticamente significativi indicano come i pazienti affetti da melanoma con più alti livelli di FORT, rispetto a quelli con basso stress ossidativo, fossero preferenzialmente in stadio IV (OR=6.68, P<0.001) e con metastasi a distanza (stadio M1c) (OR=4.87, P=0.003), e presentassero un melanoma con caratteristiche istologiche di ulcerazione (OR=2.52, P=0.017), spessore di Breslow ≥2.0 -<4.0 mm (OR=2.29, P=0.051), mitosi>1 (OR=2.16, P=0.064), e di tipo nodulare (OR=2.12, P=0.054). D'altra

parte, gli stessi pazienti erano meno frequentemente in stadio I (OR=0.31, P=0.005) e senza metastasi (linfonodali o a distanza, M0) (OR=0.15, P<0.001) e il melanoma presentava con minor frequenza spessore di Breslow <0.75 mm (OR=0.33, P=0.025), la forma superficiale (OR=0.46, P=0.043), e la variante epitelioidea (OR=0.32, P=0.014) (Tabella 3).

Tabella 3. Caratteristiche cliniche e istologiche dei 120 pazienti con melanoma e confronto tra i due sottogruppi con alti livelli di FORT ≥310 unità (n=46) e bassi livelli di FORT <310 unità (n=74).

Caratteristiche	**Tutti i casi di melanoma (n=120)**	**FORT ≥310 Unità (n=46)**	**FORT <310 Unità (n=74)**	**OR (IC) FORT ≥310 vs. <310**	**P FORT ≥310 vs. <310**
Età <50 anni, n (%)	31 (25.8)	13 (28.3)	18 (24.3)	1.23 (0.53-2.82)	0.632
Tutti i nonni nati in FVG	88 (73.3)	34 (73.9)	54 (73.0)	1.05 (0.46-2.42)	0.910
BMI ≥25 kg/m^2, n (%)	63 (52.5)	24 (52.2)	39 (52.7)	0.98 (0.47-2.04)	0.955
BMI ≥30 kg/m^2, n (%)	19 (15.8)	6 (13.0)	13 (17.6)	0.70 (0.25-2.00)	0.509
Scuole superiori, n (%)	52 (43.3)	20 (43.5)	32 (43.2)	1.01 (0.48-2.12)	0.980
Laurea, n (%)	13 (10.8)	4 (8.7)	9 (12.2)	0.69 (0.20-2.38)	0.764
MetM, n (%)	53 (44.2)	29 (63.0)	24 (32.4)	**3.55 (1.64-7.69)**	**0.001**
NMetM, n (%)	67 (55.8)	17 (37.0)	50 (67.6)	**0.28 (0.13-0.61)**	**0.001**
Stadio I, n (%)	48 (40.0)	11 (23.9)	37 (50.0)	**0.31 (0.14-0.71)**	**0.005**
Stadio II, n (%)	19 (15.8)	6 (13.0)	13 (17.6)	0.70 (0.25-2.00)	0.509
Stadio III, n (%)	33 (27.5)	14 (30.4)	19 (25.7)	1.27 (0.56-2.86)	0.570
Stadio IV, n (%)	20 (16.7)	15 (32.6)	5 (6.8)	**6.68 (2.23-20.0)**	**<0.001**
M0	100 (83.3)	31 (67.4)	69 (93.2)	**0.15 (0.05-0.45)**	**<0.001**
M1a	3 (2.5)	3 (6.5)	0 (-)	1.07 (0.99-1.15)	0.054^
M1b	0 (-)	0 (-)	0 (-)	-	-
M1c	17 (14.2)	12 (26.1)	5 (6.8)	**4.87 (1.59-14.9)**	**0.003**
Tronco, n (%)	67 (55.8)	23 (50.0)	44 (59.5)	0.68 (0.32-1.43)	0.310
Arti superiori, n (%)	8 (6.7)	2 (4.3)	6 (8.1)	0.51 (0.10-2.67)	0.709
Arti inferiori, n (%)	26 (21.7)	10 (21.7)	16 (21.6)	1.01 (0.41-2.46)	0.988
Mani/piedi, n (%)	8 (6.7)	5 (10.9)	3 (4.1)	2.89 (0.66-12.7)	0.257
Testa/collo, n (%)	11 (9.2)	6 (13.0)	5 (6.8)	2.07 (0.59-7.22)	0.331
Diffusione superficiale, n (%)	61 (50.8)	18 (39.1)	43 (58.1)	**0.46 (0.22-0.98)**	**0.043**
Nodulare, n (%)	42 (35.0)	21 (45.7)	21 (28.4)	2.12 (0.98-4.58)	0.054^
Acrale lentigginoso, n (%)	5 (4.2)	3 (6.5)	2 (2.7)	2.51 (0.40-15.6)	0.370

Lentigo maligna, n (%)	2 (1.7)	0 (-)	2 (2.7)	0.97 (0.94-1.01)	0.523
Spitzoide, n (%)	5 (4.2)	3 (6.5)	2 (2.7)	2.51 (0.40-15.6)	0.370
Altri, n (%)	7 (5.8)	1 (2.2)	6 (8.1)	0.25 (0.03-2.16)	0.248
Spessore di Breslow <0.75 mm. n (%)	29 (24.2)	6 (13.0)	23 (31.1)	**0.33 (0.12-0.89)**	**0.025**
Spessore di Breslow ≥0.75 -<1.0 mm, n (%)	11 (9.2)	5 (10.9)	6 (8.1)	1.38 (0.40-4.82)	0.747
Spessore di Breslow ≥1.0 -<2.0 mm, n (%)	34 (28.3)	15 (32.6)	19 (25.7)	1.40 (0.62-3.14)	0.413
Spessore di Breslow ≥2.0 -<4.0 mm, n (%)	30 (25.0)	16 (34.8)	14 (18.9)	2.29 (0.99-5.30)	0.051^
Spessore di Breslow ≥4.0 mm, n (%)	16 (13.3)	4 (8.7)	12 (16.2)	0.49 (0.15-1.63)	0.239
Clark I, n (%)	2 (1,7)[d]	1 (2.3)[e]	1 (1.4)	1.70 (0.10-27.8)	1.000
Clark II, n (%)	29 (24.6)[d]	7 (15.9)[e]	22 (29.7)	0.45 (0.17-1.15)	0.092^
Clark III, n (%)	23 (19.5)[d]	8 (18.9)[e]	15 (20.3)	0.87 (0.34-2.27)	0.782
Clark IV, n (%)	59 (50.0)[d]	25 (56.8)[e]	34 (45.9)	1.55 (0.73-3.28)	0.253
Clark V, n (%)	3 (2.5)[d]	2 (4.5)[e]	1 (1.4)	3.48 (0.31-39.5)	0.555
Ulcerazione, n (%)	44 (36.7)	23 (50.0)	21 (28.4)	**2.52 (1.17-5.44)**	**0.017**
Mitosi >1, n (%)	76 (64.4)[d]	33 (75.0)[e]	43 (58.1)	2.16 (0.95-4.93)	0.064^
Regressione, n (%)	15 (12.7)[d]	5 (11.4)[e]	10 (13.5)	0.82 (0.26-2.58)	0.735
TILs[c] Brisk positivo, n (%)	32 (27.1)[d]	12 (27.3)[e]	20 (27.0)	1.01 (0.44-2.34)	0.977
TILs[cn] on-brisk, n (%)	43 (36.4)[d]	17 (38.6)[e]	26 (35.1)	1.16 (0.54-2.52)	0.702
TILs[c] assente, n (%)	42 (35.6)[d]	14 (31.8)[e]	28 (37.8)	0.77 (0.35-1.69)	0.509
Microsatellitosi, n (%)	5 (4.2)[d]	3 (6.8)[e]	2 (2.7)	2.63 (0.42-16.4)	0.360
Variante epitelioide, n (%)	34 (28.8)[d]	7 (15.9)[e]	27 (36.5)	**0.32 (0.13-0.82)**	**0.014**
Variante fusata, n (%)	10 (8.4)[f]	3 (6.7)[g]	7 (9.5)	0.68 (0.17-2.79)	0.741
Variante a piccole cellule, n (%)	2 (1.7)[f]	0 (-)[g]	2 (2.7)	0.97 (0.94-1.01)	0.526
Più di 1 melanoma, n (%)	19 (15.8)	11 (23.9)	8 (10.8)	2.59 (0.96-7.04)	0.056^

[a] U-Test a due code di Mann-Whitney. [b] Non calcolabile perchè il gruppo aveva 0 soggetti. [c] TILs, infiltrato linfocitario tumorale. [d] Dati disponibili per 118 pazienti. [e] Dati disponibili per 44 pazienti. [f] Dati disponibili per 119 pazienti. [g] Dati disponibili per 45 pazienti. Le differenze significative sono state indicate in grassetto; le tendencies sono state evidenziate con il simbolo ^.

5.6 DISCUSSIONE

Nel presente lavoro vengono presentati nuovi risultati riguardanti lo stress ossidativo e la capacità antiossidante totale nei pazienti con melanoma.

L'interesse scientifico sul ruolo dello stress ossidativo nel melanoma è via via più crescente sia per quello che riguarda la prevenzione (Gruber and Halliwell 2017), sia per quanto riguarda la progressione della malattia (Bisevac et al. 2018; Cannavò et al. 2019). Nuovi studi sono quanto mai utili per fare chiarezza sulle controversie ancora esistenti in Letteratura, soprattutto per quanto riguarda l'utilizzo di strategie antiossidanti in ambito oncologico (Huang et al. 2005; Song et al. 2009; Jensen et al. 2010; Gorrini et al. 2013; Yin et al. 2013; Cattaneo et al. 2015; Knott et al. 2015; Kikuchi et al, 2019; Moyer et al. 2014; Chandel and Tuveson 2014; Glasauer and Chandel 2014; Mishra et al. 2018; Tsoi et a. 2018; Chen et al. 2019).

In generale l'ipotesi che il melanoma sia sostenuto da distress ossidativo è legata al fatto che i melanociti dell'epidermide sono particolarmente vulnerabili allo stress ossidativo derivante dallo status pro-ossidativo che si genera durante la sintesi di melanina e dalla compromissione delle difese antiossidanti intrinseche che avviene durante i processi patologici (Denat et al. 2014). Fino ad ora i meccanismi fisiopatologici dei fattori pro e anti-ossidanti nel melanoma sono stati oggetto di studio in alcuni lavori su cellule *in vitro* o su animali (Cannavò et al. 2019), ma ancora non risultano compresi fino in fondo i complessi meccanismi che guidano *in vivo* la regolazione dello stress ossidativo nei pazienti con melanoma, metastatico e non (Bisevac et al. 2018; Cannavò et al. 2019).

Riguardo ai risultati sulla perossidazione lipidica, ottenuti tramite il test FORT, il presente studio è il primo che ha analizzato i livelli ematici di stress ossidativo nei melanomi metastatici e non metastatici in una popolazione di 120 pazienti caucasici residenti in una specifica regione italiana con elevato tasso di incidenza per melanoma cutaneo. L'analisi dei valori ottenuti ha dimostrato che i livelli di stress ossidativo variano considerabilmente tra i pazienti con melanoma a seconda del grado metastatico, evidenziando come i valori ≥310 unità FORT si osservino più frequentemente tra i melanomi metastatici rispetto ai non metastatici (OR=3.55, P=0.001). L'associazione metastasi-stress ossdativo non sembra risentire del BMI, dato che non sono state rilevate differenze significative nei valori di BMI>30 e i valori di FORT. D'altra parte non si è

osservato un aumento di stress ossidativo tra i pazienti non metastatici rispetto ai controlli sani. Globalmente questi dati indicano come un elevato stress ossidativo sembra associarsi alle metastasi. Non è però possibile dedurre se le cellule metastatiche sovraproducano ROS favorendo la loro stessa proliferazione o se un ambiente pro-ossidante favorisca l'instaurarsi del processo metastatico. Un recente studio eseguito su ratti trapiantati con melanoma da 4 pazienti ha riscontrato che i livelli citoplasmatici di ROS erano significativamente più alti nelle cellule circolanti di melanoma e nei noduli metastatici viscerali rispetto alle metastasi sottocutanee. Inoltre, le metastasi viscerali mostravano livelli mitocondriali di ROS maggiori rispetto alle metastasi sottocutanee e le cellule circolanti (Piskounova et al. 2015). Sulla base di questi riscontri, l'aumento ematico dei ROS che è stato riscontrato nel presente lavoro nei pazienti metastatici, in particolar modo pazienti in stadio M1c con metastasi a distanza, potrebbe derivare dall'aumentata produzione di ROS da parte delle cellule metastatiche.

Per quanto riguarda i pochi studi su pazienti esistenti in tema stress ossidativo e melanoma, nel 2015 è stato pubblicato uno studio brasiliano che ha esaminato 43 pazienti (15 uomini e 28 donne) con melanoma cutaneo non metastatico (stadio I e II) e 50 pazienti sani (11 uomini e 39 donne) (Santos Bernardes et al. 2015). I pazienti con melanoma operato chirurgicamente mostravano alti livelli di malondialdeide (MDA), quale indicatore di perossidazione lipidica, e di tioli, bassi livelli di GSH e livelli invariati dei parametri antiossidanti totali e di acido urico rispetto ai controlli. Inoltre, lo stesso studio suggeriva una correlazione tra lo spessore di Breslow e uno status sistemico pro-ossidante, conclusione simile a quanto emerso dai risultati del presente lavoro. In precedenza, anche Gadjeva ha riscontrato un aumento significativo di MDA e catalasi e una riduzione dei livelli di SOD in 21 pazienti con melanoma, precedentemente all'asportazione chirurgica, rispetto al gruppo di controllo. Successivamente alla rimozione chirurgica del tumore, i livelli di MDA scendevano a livelli simili a quelli dei controlli sani (Gadjeva et al. 2008). Gli alti livelli di MDA misurati dopo asportazione del melanoma nello studio di Santos Bernardes, similmente a quelli di FORT del presente studio, vengono giustificati dalla probabile persistenza di infiammazione anche dopo la chirurgia (Santos Bernardes et al. 2015). Oppure un tumore più aggressivo potrebbe aver determinato più facilmente disseminazione tumorale in circolo a spiegazione del maggior livello di stress ossidativo ottenuto (Obrador et al. 2018). Il gruppo di lavoro brasiliano ha poi pubblicato un successivo studio in cui ha confrontato 30 pazienti con melanoma e 30 controlli riscontrando nei pazienti con melanoma un aumento della MDA, mentre, nei soli pazienti metastatici, un incremento di prodotti di ossidazione avanzata proteica e dei livelli di tioli. I livelli di catalasi, GSH e IL-1β erano diminuiti nei pazienti non metastatici (Santos Bernardes et al. 2016). Anche da un punto di vista immunoistochimico è stata evidenziata un'aumentata espressione tissutale di MDA nel melanoma, oltre ad un aumento dell'espressione

degli enzimi antiossidanti SOD e catalasi (Sander et al. 2003). Riguardo ai valori della capacità antiossidante totale, nel nostro campione di 240 soggetti, e in particolare nei pazienti con melanoma e nei controlli sani, è stata evidenziata una relazione inversa tra i livelli di stress ossidativo e quelli delle difese antiossidanti, così come osservato in precedenti studi (Francescato et al. 2014; Cauci et al. 2017; Lewis et al. 2016). Nel dettaglio, i pazienti con melanoma presentavano una capacità antiossidante totale ridotta (<1.1 unità FORD) rispetto ai controlli sani ed ancora più marcata era la differenza tra melanomi metastatici e pazienti sani (OR=11.4, P<0.001) che tra melanomi non metastatici e controlli sani (OR=4.18, P<0.001) La compromissione delle molecole antiossidanti nel melanoma è stata evidenziata in alcuni studi. Per esempio, un fattore chiave delle difese antiossidanti è il glutatione ridotto (GSH), necessario per la trasformazione di H_2O_2 in H_2O. In particolare, Piskounova et al. hanno riscontrato un basso rapporto tra GSH e glutatione ossidato (GSSG) nei noduli metastatici o nelle cellule circolanti di melanoma rispetto alle metastasi sottocutanee nei ratti trapiantati con melanoma (Piskounova et al. 2015). Dai risultati del presente lavoro non è possibile però fornire un nesso di causalità, cioè inferire se il melanoma aumenti direttamente la produzione di ROS che a sua volta inducono perossidazione lipidica e quindi consumo delle difese antiossidanti e/o se il melanoma riduca la capacità antiossidante rendendola insufficiente per neutralizzare i radicali liberi che a loro volta producono perossidazione lipidica. Confrontando lavori simili sull'argomento, i nostri risultati sono parzialmente concordi con la recente pubblicazione di Bisevac et al. che ha esaminato i seguenti parametri di stress ossidativo: anione radicale superossido ($O_2^{\bullet-}$), superossido dismutasi totale (tSOD) e mitocondriale (MnSOD), catalasi e MDA. Secondo i dati pubblicati, i livelli di MDA erano più elevati nei melanomi rispetto ai controlli, con i valori maggiori nei pazienti in stadio IV; l'attività della tSOD e MnSOD non differivano statisticamente confrontando 72 pazienti con melanoma e 30 soggetti sani, sebbene i pazienti in stadio III avessero più alti livelli di tSOD e quelli in stadio IV più alti livelli di MnSOD rispetto ai controlli. Al contrario, l'attività della catalasi risultava maggiore nei melanomi in stadio I, II e III, ma non nei pazienti in stadio IV rispetto ai controlli (Bisevac et al. 2018).

Di fatto la biologia dello stress ossidativo nel melanoma rimane ancora da chiarire. I dati a disposizione mostrano generalmente un aumento dei marker di perossidazione lipidica nel melanoma, mentre ci sono risultati discordanti sui livelli delle molecole antiossidanti, che in alcuni lavori, come il presente, sembrano essere ridotti, come da consumo delle difese antiossidanti conseguenti al maggior carico di stress ossidativo e in altri lavori sembrano aumentate, come da iperattivazione della risposta antiossidante nei confronti dei ROS. La diversità di risultati potrebbe anche dipendere dal tipo di misurazione effettuata dato che alcuni Autori hanno dosato i livelli di antiossidanti enzimatici (es: SOD, catalasi e GPX) e altri gli antiossidanti non enzimatici, come nel

caso del test FORD. Inoltre, sembra che il bilancio redox delle cellule varii con la progressione del tumore (Obrador et al. 2018).

Una possibile spiegazione generale viene data dalla recente review pubblicata dal gruppo italiano di Cannavò et al. sul ruolo dello stress ossidativo nella biologia del melanoma. La review ha esaminato 29 studi concludendo come i ROS siano aumentati nelle cellule tumorali e abbiano un duplice effetto: da un lato quello di promuovere la sopravvivenza del tumore, la proliferazione e la metastatizzazione, dall'altro, a livelli eccessivi, quello di indurre apoptosi e senescenza tumorale attraverso un danno al DNA. In questo senso, il melanoma sembrerebbe possedere dei meccanismi adattativi per superare gli effetti degli alti livelli di ROS (Cannavò et al. 2019). Infatti, nelle cellule sane i ROS sono causa di instabilità genomica e di danno cellulare, mentre nelle cellule cancerose lo status ossidativo è peculiare ed i ROS assumono un ruolo di molecole segnale favorenti i meccanismi di proliferazione cellulare, angiogenesi e metastatizzazione. Il bilancio redox delle cellule tumorali deve però essere attentamente regolato. Infatti, i ROS hanno anche un effetto anti-tumorogenico ed un loro livello eccessivo può indurre morte cellulare. Per mantenere i ROS ad un livello ottimale che permetta l'attivazione del pathway pro-tumorogenico senza indurre morte cellulare, le cellule aumentano la loro capacità antiossidante. Durante tutte le fasi della progressione tumorale (proliferazione, sopravvivenza e metastasi), l'omeostasi ossidativa deve essere finemente bilanciata mediante l'attivazione di diverse molecole specifiche per le diverse fasi (Reczek et al. 2017). Molto recentemente Tasdogan et al. hanno anche provato come alterate funzioni di molecole coinvolte nella regolazione dello stress ossidativo generino tra le cellule cancerose del melanoma differenze metaboliche in grado di influire sul potenziale metastatico (Tasdogan et al. 2020).

Sulla base di queste ipotesi si basano le strategie terapeutiche in corso di studio che inizialmente erano dirette a ridurre il carico ossidativo mediante l'uso di antiossidanti, più recentemente si stanno indirizzando verso l'approccio pro-ossidante per alterare l'omeostasi redox spostandone l'equilibrio verso la morte cellulare indotta da eccessivo stress ossidativo (Obrador et al. 2018).

I risultati in Letteratura non sono ancora concordi, oltretutto gli effetti anti-tumorali o pro-tumorali delle diverse molecole sembrano dipendere dalla loro struttura chimica, dalla dose utilizzata e dalla concentrazione raggiunta in vivo. Ad esempio, sembra che l'utilizzo di integratori antiossidanti con la dieta non abbia il risultato sperato sulla riduzione dell'incidenza di tumore in quanto essi agirebbero con un'inibizione dei ROS a distanza dal loro sito di produzione cellulare e non contrastatando il segnale pro-tumorogenico indotto dai ROS a livello locale. Quest'ultima azione si potrebbe invece raggiungere con l'utilizzo di antiossidanti a bersaglio mitocondriale, sede elettiva della produzione di ROS coinvolti nei segnali pro-tumorali (Chandel et al. 2014).

Sicuramente una maggiore comprensione dei meccanismi molecolari dello stress ossidativo nel melanoma e l'identificazione di molecole target potrebbe portare allo sviluppo di strategie mirate ad aumentare le difese antiossidanti contro il melanoma. A tale scopo, nell'ottica della medicina personalizzata, la valutazione del bilancio ossidativo in vivo di un dato paziente, associato all' identificazione di biomarcatori genetici e di fattori di rischio comportamentali, permetterebbe di formulare degli integratori mirati per le caratteristiche di ogni singolo paziente, sia in ottica preventiva che terapeutica, oltre che guidare il corretto stile di vita del paziente.

5.7 CONCLUSIONI

I risultati del presente lavoro confermano un'influenza dello stress ossidativo sul rischio di melanoma cutaneo, soprattutto metastatico.

Al di là dell'esposizione ai raggi UV (soprattutto se intermittente e con scottature solari), l'evidenza crescente dimostra l'esistenza di altri fattori implicati nell'omeostasi dello stress ossidativo che potrebbero giocare un ruolo importante nel melanoma cutaneo. Tra questi si annoverano la pelle chiara (associata a bassi livelli di eumelanina), la risposta infiammatoria dell'ospite, l'omeostasi della vitamina D, l'esposizione a inquinanti ambientali, il fumo di sigaretta, le abitudini di vita e il background genetico (Newton-Bishop et al. 2015; Hardie et al. 2019; IARC; La Marra et al. 2017; Cauci et al. 2017; Cauci et al. 2019; Zmijewski MA 2019).

A tal proposito, è interessante notare come in un precedente lavoro (Cauci et al. 2017) sia stato riscontrato come l'obesità fosse 8 volte più frequente nei pazienti con melanoma metastatico rispetto ai soggetti sani e ci fosse anche una correlazione tra obesità, fumo, polimorfismi di VDR e metastasi. Nel presente lavoro, al contrario, non è stata dimostrata una differenza significativa dei valori di BMI tra pazienti con valori elevati e bassi di FORT, come se l'obesità influisse sulle metastasi, ma non sullo stress ossidativo. La Letteratura di fatto, asserirebbe invece una relazione proporzionale tra obesità e stress ossidativo, mediata dal contesto infiammatorio (Oliveira et. 2016).

I dati del presente lavoro sono probabilmente limitati dal fatto che non è stata eseguita una analisi multivariata e necessitano di ulteriori approfondimenti, tuttavia è anche possibile che i fattori scatenanti lo stress ossidativo e la riduzione delle difese antiossidanti nei pazienti con melanoma siano soverchianti rispetto agli effetti dovuti al BMI.

Un ruolo di rilievo nella regolazione del bilancio redox che merita di essere ancora di più approfondito è anche quello della vitamina D a cui viene riconosciuta un'attività antiossidante diretta (Philips et al. 2019). Essendo un ormone pleiotropico, le sue azioni sono molteplici e si

esplicano non solo a livello del metabolismo calcio-fosforo, ma anche come vitamina immunomodulante e anticarcinogenetica (Zmijewski MA 2019). E' stato per esempio dimostrato che bassi livelli di vitamina D si associno a melanomi con maggiore spessore di Breslow (Caini et al. 2014). Ammettendo un ruolo dello stress ossidativo nella tumorogenesi, la vitamina D sembra agire attraverso il suo recettore VDR nella regolazione della respirazione mitocondriale, proteggendo la cellula dal danno conseguente all'eccessivo accumulo di ROS (Ricca et al. 2018). L'integrazione dei risultati sullo stress ossidativo con quelli sui polimorfismi del VDR nel melanoma potrebbe fornire maggiori informazioni e porre le basi per ulteriori studi in merito all'utilizzo di supplementi dietetici basati sulle caratteristiche genetiche e di status ossidativo del paziente.

Un migliore conoscenza degli aspetti molecolari e genetici del melanoma avrebbe un risvolto anche in campo terapeutico, soprattutto nell'ambito della malattia metastatica, dove gli sforzi scientifici sono sempre più diretti allo sviluppo di terapie a bersaglio molecolare. In particolare, il panorama terapeutico attuale prevede l'utilizzo della terapia target anti-BRAF, in caso di evidenza della mutazione del gene, o, in caso di fallimento della prima o assenza della mutazione, la somministrazione di immunoterapia con anticorpi monoclonali diretti contro l'antigene 4 associato ai linfociti T citotossici (CTL4-A) o verso la proteina 1 della morte cellulare programmata (PD-1). Nonostante queste terapie abbiano completamente cambiato la prognosi del melanoma avanzato, rimane il problema dello sviluppo di resistenza alla terapia. In questo senso i maggiori sforzi sono volti ad identificare biomarcatori di aiuto nella selezione di uno specifico trattamento per un dato paziente oltre che i meccanismi molecolari alla base della resistenza primaria e secondaria (Luke et al. 2017). Ad esempio, per ovviare allo sviluppo di resistenza nei confronti degli inibitori di BRAF, dato che tali farmaci sembrano aumentare i livelli di ROS nelle cellule di melanoma, è stato studiata l'associazione con un pro-framaco che viene attivato dall'elevata quota di ROS (Yuan et al. 2018). Tale campo di studio è quanto mai aperto e la valutazione di marcatori individuali che possano personalizzare il percorso diagnostico-terapeutico ed essere utilizzati anche in ottica preventiva è sempre più accattivante.

Inserendosi in un contesto di medicina personalizzata, i dati emersi dagli studi del nostro gruppo di lavoro pubblicati negli ultimi tre anni (Cauci et al. 2017; La Marra et al. 2017; Cauci et al. 2019) hanno indagato nel complesso vari aspetti molecolari implicati nel melanoma e hanno portato all'identificazione di possibili fattori di rischio genetici e ambientali e all'individuazione di possibili biomarcatori. I risultati relativi allo stress ossidativo arrichiscono i dati ottenuti finora indicando possibili nuove strategie di prevenzione del melanoma e aprono la strada, con il supporto della più recente Letteratura (Philips et al. 2020), per nuove ricerche mirate a verificare una possibile correlazione tra gli aspetti genetici che regolano il sistema endocrino della vitamina

D e lo stress ossidativo nel melanoma, così come il ruolo del bilancio ossidativo sull'efficacia delle attuali e future terapie per il melanoma, in particolar modo avanzato.

6. BIBLIOGRAFIA

Balch CM, Gershenwald JE, Soong SJ, Thompson JF, Atkins MB, Byrd DR et al. F*inal version of 2009 AJCC melanoma staging and classification.* J Clin Oncol. 2009; 27(36): 6199–206.

Bisevac JP, Djukic M, Stanojevic I, Stevanovic I, Mijuskovic Z, Djuric A, et al. *Association between oxidative stress and melanoma progression.* J Med Biochem. 2018;37(1):12-20.

Caini S, Boniol M, Tosti G, Magi S, Medri M, Stanganelli I, et al. *Vit D and melanoma and non-melanoma skin cancer and prognosis: A comprehensive review and meta-analysis.* Eur J Cancer. 2014; 50:2649-58.

Cannavò SP, Tonacci A, Bertino L, Casciaro M, Borgia F, Gangemi S. *The role of oxidative stress in the biology of melanoma: A systematic review.* Pathol Res Pract. 2019; 215(1):21-8.

Carvalho AN, Firuzi O, Gama MJ, Horssen JV, Saso L. *Oxidative Stress and Antioxidants in Neu rological Diseases: Is There Still Hope?*. Curr Drug Targets. 2017; 18(6):705-18.

Cassidy PB, Grossman D, Leachman S. *Melanoma prevention. In: Emerging Therapeutics for Melanoma.* Edited by Marincola FM, Ascierto PA, Kirkwood JM. London, Future Medicine Ltd 2012: 30-42.

Cattaneo L, Cicconi R, Mignogna G, Giorgi A, Mattei M, Graziani G, et al. *Anti-Proliferative effect of Rosmarinus officinalis L. extract on human melanoma A375 Cells.* PLoS One. 2015;10(7):e0132439.

Cauci S, Buligan C, Marangone M, Francescato MP. *Oxidative stress in female athletes using combined oral contraceptives*. Sports Med Open. 2016;2(1):40.

Cauci S, Maione V, Buligan C, Linussio M, Serraino D, Stinco G. *BsmI (rs1544410) and FokI (rs2228570) vitamin D receptor polymorphisms, smoking, and body mass index as risk factors of cutaneous malignant melanoma in northeast Italy.* Cancer Biol Med. 2017;14(3):302-318.

Cauci S, Buligan C, Rocchi F, Salvador I, Xodo L, Stinco G. *Interleukin1 receptor antagonist gene variable number of tandem repeats polymorphism and cutaneous melanoma.* Oncol Lett. 2019; 18(6):5759-68.

Chandel NS, Tuveson DA. *The promise and perils of antioxidants for cancer patients.* N Engl J Med. 2014; 371:177–8.

Chen XY, Chen Y, Qu CJ, Pan ZH, Qin Y, Zhang X, Liu WJ, Li DF, Zheng Q. *Vitamin C induces human melanoma A375 cell apoptosis via Bax- and Bcl-2-mediated mitochondrial pathways*. Oncol Lett. 2019;18(4):3880-6.

Cecconi L, Busolin A, Barbone F, Serraino D, Chiarugi A, Biggeri A, Catelan D. *Spatial analysis of incidence of cutaneous melanoma in the Friuli Venezia Giulia region in the period 1995-2005.* Geospat Health. 2016; 11(1):422.

Crotty GF, Ascherio A, Schwarzschild MA. *Targeting urate to reduce oxidative stress in Parkinson disease.* Exp Neurol. 2017; 298:210-224.

Dal Negro RW, Visconti M, Micheletto C, Pomari C, Squaranti M, Turati C, et al. *Normal Values and Reproducibility of the Major Oxidative Stress obtained thanks to FORM System*. Italian Journal of Chest Disease. 2003; 57(3): 199-209.

Denat L, Kadekaro AL, Marrot L, Leachman SA, Abdel-Malek ZA. *Melanocytes as instigators and victims of oxidative stress.* J Invest Dermatol. 2014;134(6):1512-8.

Fischer GM, Vashisht Gopal YN, McQuade JL, Peng W, DeBerardinis RJ, Davies MA. *Metabolic strategies of melanoma cells: Mechanisms, interactions with the tumor microenvironment, and therapeutic implications.* Pigment Cell Melanoma Res. 2018;31(1):11-30.

Fitzpatrick TB. *The validity and practicality of sun-reactive skin types I through VI*. Arch Dermatol. 1988;124(6):869-871.

Francescato MP, Stel G, Geat M, Cauci S. *Oxidative stress in patients with type 1 diabetes mellitus: is it affected by a single bout of prolonged exercise?* PLoS One. 2014;9(6):e99062.

Gadjeva V, Dimov A, Georgieva NJ. *Influence of therapy on the antioxidant status in patients with melanoma.* Clin Pharm Ther. 2008; 33(2): 179-85.

Glasauer A, Chandel NS. *Targeting antioxidants for cancer therapy.* Biochem Pharmacol. 2014;92(1):90-101.

Goh J, Enns L, Fatemie S, Hopkins H, Morton J, Pettan-Brewer C, Ladiges W. *Mitochondrial targeted catalase suppresses invasive breast cancer in mice.* BMC Cancer. 2011; 11:191.

Gorrini C, Harris IS, Mak TW. *Modulation of oxidative stress as an anticancer strategy*. Nat Rev Drug Discov. 2013;12(12):931-47.

Gruber J, Halliwell B. *Approaches for extending human healthspan: from antioxidants to healthspan pharmacology.* Essays Biochem. 2017; 61(3):389-99.

Gruppo di lavoro AIOM - AIRTUM - Fondazione AIOM. *I numeri del cancro in Italia 2017*. Roma: Ed. Il pensiero scientifico editore, 2017.

Gruppo di lavoro AIOM - AIRTUM - Fondazione AIOM – PASSI - PASSI D'Argento – SIAPEC-IAP. *I numeri del cancro in Italia 2019*. Brescia: Ed. Intermedia editore, 2019.

Guy GP Jr, Thomas CC, Thompson T, Watson M, Massetti GM, Richardson LC; Centers for Disease Control and Prevention (CDC). *Vital signs: melanoma incidence and mortality trends and projections-United States, 1982-2030.* MMWR Morb Mortal Wkly Rep. 2015; 64(21):591-6.

Halliwell B. *Free radicals and antioxidants: updating a personal view.* Nutr Rev. 2012; 70(5):257-65.

Hardie CM, Elliott F, Chan M, Rogers Z, Bishop DT, Newton-Bishop JA. *Environmental exposures such as smoking and low vitamin D are predictive of poor outcome in cutaneous melanoma rather than other deprivation measures.* J Invest Dermatol. 2019 Aug 16. pii: S0022-202X(19)32703-4.

Harma MI. *The FORT test:* Reply to dr Harma and colleagues. Abramson JL. The FORT test: A novel oxidative stress marker or a well-known measure of ceruloplasmin oxidase activity?. Atherosclerosis. 2006; 187: 441-4.

https://ecis.jrc.ec.europa.eu/ (ultimo accesso 28/2/2020)

Huang SC, Ho CT, Lin-Shiau SY, Lin JK. C*arnosol inhibits the invasion of B16/F10 mouse melanoma cells by suppressing metalloproteinase-9 through down-regulating nuclear factor- kappa B and c-Jun.* Biochem Pharmacol. 2005;69(2):221-32.

IARC. International Agency for Research on Cancer. http://monographs.iarc.fr/ENG/Classification/. (ultimo accesso 28/2/2020).

Jensen JD, Wing GJ, Dellavalle RP. *Nutrition and melanoma prevention.* Clin Dermatol. 2010; 28(6), 644-9.

Jones D.P. *Redefining oxidative stress, Antioxidants & Redox Signaling*. 2006; 8:1865–79.

Jones D.P. *Radical-free biology of oxidative stress*. American Journal of Physiology—Cell Physiology. 2008; 295(4):849–68.

Kadekaro AL, Chen J, Yang J, Chen S, Jameson J, Swope VB, et al. *Alpha-melanocyte-stimulating hormone suppresses oxidative stress through a p53-mediated signaling pathway in human melanocytes.* Mol Cancer Res. 2012; 10(6):778-86.

Kamhieh-Milz J, Salama A. *Oxidative stress is predominant in female but not in male patients with autoimmune thrombocytopenia.* Oxid Med Cell Longev. 2014:720347.

Kikuchi H, Yuan B, Hu X, Okazaki M. *Chemopreventive and anticancer activity of flavonoids and its possibility for clinical use by combining with conventional chemotherapeutic agents.* Am J Cancer Res. 2019;9(8):1517-35.

Kim Y, He YY. *Ultraviolet radiation-induced non-melanoma skin cancer: Regulation of DNA damage repair and inflammation.* Genes Dis. 2014;1(2):188-198.

Klein EA, Thompson IM Jr, Tangen CM, Crowley JJ, Lucia MS, Goodman PJ, et al. *Vitamin E and the risk of prostate cancer: the Selenium and Vitamin E Cancer Prevention Trial (SELECT).* JAMA. 2011;306(14):1549-56.

Knott A, Achterberg V, Smuda C, Mielke H, Sperling G, Dunckelmann K, et al. T*opical treatment with coenzyme Q10-containing formulas improves skin's Q10 level and provides antioxidative effects.* Biofactors. 2015;41(6):383-90.

Koh HK, Geller AC, Miller DR, Grossbart TA, Lew RA. *Prevention and early detection strategies for melanoma and skin cancer - Current status.* Arch Dermatol. 1996;132(4):436–443.

La Marra F, Stinco G, Buligan C, Chiriacò G, Serraino D, Di Loreto C, et al. *Immunohistochemical evaluation of vitamin D receptor (VDR) expression in cutaneous melanoma tissues and four VDR gene polymorphisms.* Cancer Biol Med. 2017;14(2):162-175.

Le Gal K, Ibrahim MX, Wiel C, Sayin VI, Akula MK, et al. 2015. *Antioxidants can increase melanoma metastasis in mice.* Sci. Transl. Med. 7:308.

Lewis NA, Newell J, Burden R, Howatson G, Pedlar CR. *Critical difference and biological variation in biomarkers of oxidative stress and nutritional status in athletes.* PLoS One. 2016;11(3):e0149927.

Lichtenberg D, Pinchuk I. *Oxidative stress, the term and the concept.* Biochem Biophys Res Commun. 2015;461(3):441–4.

Liu F, Gomez Garcia AM, Meyskens FL Jr. *NADPH oxidase 1 overexpression enhances invasion via matrix metalloproteinase-2 and epithelial-mesenchymal transition in melanoma cells.* J Invest Dermatol. 2012;132(8):2033-41.

Lopes AC, Peixe TS, Mesas AE, Paoliello MM. *Lead exposure and oxidative stress: A systematic review.* Rev Environ Contam Toxicol. 2016; 236:193-238.

Luke JJ, Flaherty KT, Ribas A, Long GV. *Targeted agents and immunotherapies: optimizing outcomes in melanoma.* Nat Rev Clin Oncol. 2017; 14(8):463-482.

Micek A, Godos J, Lafranconi A, Marranzano M, Pajak A. *Caffeinated and decaffeinated coffee consumption and melanoma risk: a dose-response meta-analysis of prospective cohort studies.* Int J Food Sci Nutr. 2018;69(4):417-26.

Mishra H., Mishra PK, Ekielski A, Jaggi M, Iqbal Z, Talegaonkar S. *Melanoma treatment: from conventional to nanotechnology.* J Cancer Res Clin Oncol. 2018; 144(12):2283-2302.

Miura K, Green AC. *Dietary antioxidants and melanoma: evidence from cohort and intervention studies.* Nutr Cancer. 2015; 67: 867–76.

Moyer VA, U.S. Preventive Services Task Force. *Vitamin, mineral, and multivitamin supplements for the primary prevention of cardiovascular disease and cancer: U.S. Preventive services Task Force recommendation statement.* Ann Intern Med. 2014;160(8):558-64.

Newton-Bishop JA, Davies JR, Latheef F, Randerson-Moor J, Chan M, Gascoyne J, et al. *25-Hydroxyvitamin D2 /D3 levels and factors associated with systemic inflammation and melanoma survival in the Leeds Melanoma Cohort*. Int J Cancer. 2015;136(12):2890-9.

Obrador E, Liu-Smith F, Dellinger RW, Salvador R, Meyskens FL, Estrela JM. *Oxidative stress and antioxidants in the pathophysiology of malignant melanoma.* Biol Chem. 2019; 400(5):589-612.

Oliveira S, Coelho P, Prudêncio C, Vieira M, Soares R, Guerreiro SG, et al. *Melanoma and obesity: Should antioxidant vitamins be addressed?*. Life Sci. 2016; 165:83-90.

Omenn GS, Goodman GE, Thornquist MD, Balmes J, Cullen MR, Glass A, et al. *Risk factors for lung cancer and for intervention effects in CARET, the Beta-Carotene and Retinol Efficacy Trial.* J. Natl. Cancer Inst. 1996; 88, 1550–9.

Palmieri B, Sblendorio V. *Oxidative stress detection: what for? Part I.* Eur Rev Med Pharmacol Sci. 2006; 10(6): 291-317.

Palmieri B, Sblendorio V. *Oxidative stress tests: overview on reliability and use*. *Part II*. Eur Rev Med Pharmacol Sci. 2007; 11: 383-99.

Philips N, Ding X, Kandalai P, Marte I, Krawczyk H, Richardson R. *The Beneficial Regulation of Extracellular Matrix and Heat Shock Proteins, and the Inhibition of Cellular Oxidative Stress Effects and Inflammatory Cytokines by 1,25-dihydroxyvitamin D3 in Non-Irradiated and Ultraviolet Radiated Dermal Fibroblasts*. Cosmetics. 2019; 6: 46.

Philips N, Samuel P, Keller T, Alharbi A, Alshalan S, Shamlan SA. *Beneficial Regulation of Cellular Oxidative Stress Effects, and Expression of Inflammatory, Angiogenic, and the Extracellular Matrix Remodeling Proteins by 1α,25-Dihydroxyvitamin D3 in a Melanoma Cell Line.* Molecules. 2020: 5;25(5).

Picardo M, Grammatico P, Roccella F, Roccella M, Grandinetti M, Del Porto G, et al. *Imbalance in the antioxidant pool in melanoma cells and normal melanocytes from patients with melanoma.* J Invest Dermatol. 1996;107(3):322-6.

Pignatelli P, Menichelli D, Pastori D, Violi F. *Oxidative stress and cardiovascular disease: new insights.* Kardiol Pol. 2018; 76(4):713-22.

Piskounova E, Agathocleous M, Murphy MM, Hu Z, Huddlestun SE, Zhao Z, et al. *Oxidative stress inhibits distant metastasis by human melanoma cells. N*ature. 2015;527(7577):186-91.

Porporato PE, Payen VL, Pérez-Escuredo J, De Saedeleer CJ, Danhier P, Copetti T, et al. *A mitochondrial switch promotes tumor metastasis*. Cell Rep. 2014;8(3):754-66.

Reczek CR, Chandel NS. *The two faces of reactive oxygen species in cancer.* Ann. Rev. Cancer Biol. 2017; 1: 79–98.

Ricca C, Aillon A, Bergandi L, Alotto D, Castagnoli C, Silvagno F. *Vitamin D Receptor Is Necessary for Mitochondrial Function and Cell Health.* Int J Mol Sci. 2018; 19:1672.

Salhi A, Jordan AC, Bochaca II, Izsak A, Darvishian F, Houvras Y, et al. *Oxidative phosphorylation promotes primary melanoma invasion.* Am J Pathol. 2020.

Sample A, He YY. *Mechanisms and prevention of UV-induced melanoma.* Photodermatol Photoimmunol Photomed. 2018;34(1):13–24.

Sander CS, Hamm F, Elsner P, Thiele JJ. *Oxidative stress in malignant melanoma and non-melanoma skin cancer.* Br J Dermatol. 2003;148(5):913-22.

Sander CS, Chang H, Hamm F, Elsner P, Thiele JJ. *Role of oxidative stress and the antioxidant network in cutaneous carcinogenesis*. Int J Dermatol. 2004; 43(5):326-35.

Sanders LM, Henderson CE, Hong MY, Barhoumi R, Burghardt RC, Carroll RJ, et al. *Pro-oxidant environment of the colon compared to the small intestine may contribute to greater cancer susceptibility*. Cancer Lett. 2004; 208(2):155-61.

Santos Bernardes S, de Souza-Neto FP, Ramalho LN, Derossi DR, Guarnier FA, da Silva CF, et al. *Systemic oxidative profile after tumor removal and the tumor microenvironment in melanoma patients*. Cancer Lett. 2015;361(2):226-32.

Santos Bernardes S, de Souza-Neto FP, Pasqual Melo G, Guarnier FA, Marinello PC, Cecchini R, et al. *Correlation of TGF-β1 and oxidative stress in the blood of patients with melanoma: a clue to understanding melanoma progression?* Tumour Biol. 2016;37(8):10753-61.

Sies H. *Oxidative Stress.* London: Ed. Academic Press, 1985, 1–507.

Sies H. *Oxidative stress: a concept in redox biology and medicine*. RedoxBiology. 2015; 4: 180-3.

Sies H. *Hydrogen peroxide as a central redox signaling molecule in physiological oxidative stress: Oxidative eustress.* Redox Biol. 2017; 11:613-9.

Sies H, Berndt C, Jones DP. *Oxidative stress.* Annu Rev Biochem. 2017; 86:715-48.

Song X, Mosby N, Yang J, Xu A, Abdel-Malek Z, Kadekaro AL. *Alpha-MSH activates immediate defense responses to UV-induced oxidative stress in human melanocytes.* Pigment Cell Melanoma Res. 2009; 22(6):809-18.

Stadler R. *The effect of smoking in melanoma outcome still remains an enigma.* J Eur Acad Dermatol Venereol. 2019; 33(12):2219-20.

Stanganelli I, De Felici MB, Mandel VD, Caini S, Raimondi S, Corso F, et al. *The association betweeen pesticide use and cutaneous melanoma: a systematic review and meta-analysis.* J Eur Acad Dermatol Venereol. 2020; 34:691-708.

Strömberg U, Peterson S, Holmberg E, et al. *Cutaneous malignant melanoma shows geographic and socioeconomic disparities in stage at diagnosis and excess mortality.* Acta Oncol. 2016; 3:1-8.

Tasdogan A, Faubert B, Ramesh V, Ubellacker JM, Shen B, Solmonson A, et al. *Metabolic heterogeneity confers differences in melanoma metastatic potential*. Nature. 2020; 577(7788):115-20.

Torri C. *The FORD assay: a preliminary study*. 2006. Callegari SpA.

Tsoi J, Robert L, Paraiso K, Galvan C, Sheu KM, Lay J, et al. *Multi-stage differentiation defines melanoma subtypes with differential vulnerability to drug-induced Iron-dependent oxidat ive stress.* Cancer Cell. 2018; 33(5):890-904.

Venza M, Visalli M, Beninati C, De Gaetano GV, Teti D, Venza I. *Cellular mechanisms of oxidat-ive stress and action in melanoma.* Oxid Med Cell Longev. 2015; 2015:481782.

www.tumori.net (ultimo accesso 28/2/2020).

Yeum KJ, Russell RM, Krinsky NI, Aldini G. *Biomarkers of antioxidant capacity in the hydrophilic and lipophilic compartments of human plasma.* Arch Biochem Biophys. 2004; 430(1):97-103.

Yin Y, Li W, Son YO, Sun L, Lu J, Kim D, et al. *Quercitrin protects skin from UVB-induced oxidative damage.* Toxicol Appl Pharmacol. 2013; 269(2):89-99.

Yuan L, Mishra R, Patel H, Abdulsalam S, Greis KD, Kadekaro AL, et al. *Utilization of Reactive Oxygen Species Targeted Therapy to Prolong the Efficacy of BRAF Inhibitors in Melanoma.* J Cancer. 2018; 9(24):4665-76.

Zimta AA, Schitcu V, Gurzau E, Stavaru C, Manda G, Szedlacsek S, et al. *Biological and molecular modifications induced by cadmium and arsenic during breast and prostate cancer development.* Environ Res. 2019; 178:108700.

Zmijewski MA. *Vitamin D and Human Health*. Int J Mol Sci. 2019; 20(1):145.

7. APPENDICE

Polimorfismi e Melanoma

Data……

Cognome / Nome (in stampatello)

……

Data e luogo di nascita

……

Contatto per eventuali **comunicazioni** (**email , telefono, indirizzo**)

Nazionalità …… **Peso/ Altezza** ……kg ……cm

Gruppo etnico Bianco ❑ Asiatico ❑ Nero ❑ Misto specificare……

Città di residenza, indirizzo, attuali ……

……

Da che anno vive a questo indirizzo?……

Dove **vive attualmente: montagna, mare, collina, pianura** ……

……

Ha vissuto nello **stesso luogo durante l'infanzia** (0-14 anni)?..... ❑ Si ❑ No

Se ha abitato in passato in luoghi diversi da quello attuale,

SPECIFICARE dove e quando

Città di residenza, via (**in passato**) da anno ……..a anno……

……

Città di residenza, via (**in passato**) da anno …… a anno……

……

Città di residenza, via (**in passato**) da anno …… a anno……

……

Parentela luogo di nascita (ossia paese di origine, residenza):

Padre ❑ FVG (paese……) ❑ Altra regione (dove ……

Madre ❑ FVG (paese ……) ❑ Altra regione (dove ……

Nonna materna ❑ FVG (paese ……) ❑ Altra regione (dove ……

Nonno materno ❑ FVG (paese ……) ❑ Altra regione (dove …….

Nonna paterna ❑ FVG (paese ……) ❑ Altra regione (dove ……

Nonno paterno ❑ FVG (paese ……) ❑ Altra regione (dove ……

Grado di istruzione…….. Destrimane/mancino……

Tipo lavoro (o studio) **attuale** ……

……

Luogo di lavoro (o studio) **attuale** ……

Se in pensione indicare anno di pensionamento……

indicare ultimo tipo di attività lavorativa svolta prima del pensionamento
……

Se **in pensione ma continua a svolgere un lavoro** indicare il lavoro prevalente attualmente svolto
……..

Luogo lavoro ……..

In passato ha lavorato Tipo di lavoro …….. Luogo lavoro……..Da anno ……..ad anno ……

In passato ha lavorato Tipo di lavoro …….. Luogo lavoro……..Da anno ……..ad anno ……

Ha avuto figli? No ❑ **Si** ❑

Quanti figli maschi…….. Quante figlie femmine ……..

Aborti No ❑ Si ❑ numero aborti ……..

è **Astemio** ❑ **Beve alcool** ❑

❑ alcool solo nei fine settimana o occasioni speciali specificare quando
……

❑ Ogni giorno

Quanti bicchieri di **vino** (o simili, spritz. ecc)/**giorno** ……

Quanti bicchieri di **birra /settimana** ……

Beve superalcolici come grappa, whisky, amari, ecc. ? ❑

Quanti bicchieri di **superalcolici /settimana** ……..

A che età ha iniziato a bere alcool……..

Ricorda che età aveva alla prima sbornia?.....

Sa dire circa quante ubriacature importanti ha avuto nella sua vita?.....

Quante tazzine di **caffè/giorno** …… Quante tazze di **the**/giorno ……

Quante tazze di **latte/giorno** *(oppure specificare i litri)*……

Quale tipo e marca di latte acquista prevalentemente?……

Solo latte prodotto in FVG o altro specificare? ……

Quanti **yogurt/settimana**……

Quale tipo e marca di yogurt acquista?……

Porzioni di **formaggio da 50 grammi** (mezzo etto) **al giorno** ……

Quale tipo (mucca, capra, pecora, ecc.) e marca e tipo di formaggio (ad esempio montasio, stracchino, grana, ecc.) acquista prevalentemente?……

……

Prevalentemente (o solo) formaggio prodotto in FVG o di altra provenienza ? specificare
……..

Porzioni di **frutta fresca** (200 g circa) **al giorno** ……

……..

Porzioni di **verdura** (circa 50 grammi) **al giorno** specificare tipo e se prevalentemente cruda o cotta
……..

……

Porzioni di **carne rossa/settimana** (100 g circa)……

Porzioni di **carne bianca/settimana** (100 g circa)……

Mangia carne rossa cruda? ☐ Si ☐ No **Mangia carne rossa poco cotta?** ☐ Si ☐ No

Quanto spesso mangia carne rossa cruda? Ogni settimana ☐ **1-2 volte al mese** ☐ **occasionalmente** ☐

Porzioni di **insaccati** (circa 50 grammi)/**settimana**, specificare tipo (salame, prosciutto crudo, cotto, pancetta, ossacollo, salsicce)……

……

……

Porzione di **pesce/settimana**(100 g circa) specificare se pesce fresco o in scatola (tonno, sardine, ecc.)……

……

Porzione di **crostacei o molluschi/settimana**(100 g circa)……

Quante **uova/settimana**……..

Prevalentemente quale tipo di uova consuma (da negozio, da produzione propria, ecc.) ?
……..

Quanti dolci (brioche, fette di torta, porzioni da 6 biscotti, ecc./**settimana**……

specificare ……

Prevalentemente sono dolci acquistati in negozio, o da produzione casalinga?

……

Per cucinare utilizza prevalentemente (se usa sia burro che olio segnare entrambi)

Burro ❑ **Olio di oliva extravergine** ❑ **Olio di oliva** ❑ **Olio di semi** ❑

Il burro che usa è di solito friulano ❑ o di altre origini ❑

Attualmente fa uso di **integratori alimentari** (vitamine, proteine, sali minerali, ecc)?

❑ Si ❑ No

Se si, SPECIFICARE tipo e dosaggio di **integratore usato attualmente** e da quanto tempo

……

……

Se ha fatto uso in passato di integratori alimentari SPECIFICARE tipo e dosaggio di **integratori usati da anno ……..a anno……..**

……

integratori usati da anno ……..a anno……..

……

Fumatore ? ❑ Si ❑ No

Se è fumatore/fumatrice, **da quando** fuma ? ……..

Quale tipo di fumo (sigarette, pipa, altro)……

Se è fumatore/fumatrice **quante sigarette** fuma in un giorno ?.....

Ex fumatore: da quanto tempo ha smesso……..

da (anno) …….. a anno…… n. sigarette/giorno ……..

da (anno) …….. a anno…… n. sigarette/giorno ……..

da (anno) …….. a anno …… n. sigarette/giorno ……

Complessivamente per quanti anni fa fumato nella sua vita? ……

Fumo passivo ❑ No ❑ Si specificare ……..

Vive (o ha vissuto) **e/o lavora** (o ha lavorato) vicino a **fonti di inquinamento (discariche, centrali elettriche, inceneritori, forti campi elettromagnetici, fabbriche chimiche** o altro tipo, ecc.)
SPECIFICARE……..

……

……

……

Ha contatto o utilizza (o ha utilizzato in passato) con sostanze come **vernici, solventi, diserbanti, pesticidi, amianto, fumi o altre sostanze tossico/nocive, metalli,**

☐ No ☐ **Si** **Specificare tipo di sostanza, tipo di contatto, per quanto tempo**

……

……

……

……Utilizza o ha utilizzato in passato **tinture per capelli?** ☐ No ☐ **Si**

Specificare tipo di colorazione Biondo ☐ Castano ☐ Nero ☐

Per quanti anni? ……..da anno……a anno……..

Con frequenza mensile ☐ ogni 2 mesi ☐ ogni 3 mesi ☐ 2 o 3 volte all'anno ☐

Soffre o ha sofferto di **malattie gravi** (tumori, artriti/artrosi, malattie polmonari gravi, diabete, infarto, ictus, ipertensione, epatiti, problemi tiroidei o altre malattie gravi) ? ☐ Si ☐ No

SPECIFICARE quali malattie……

……
……

Ha malattie virali (come l'HIV) o che compromettono l'immunità? ☐ Si ☐ No

Se si quali? (epatite C o B, HPV, herpes, altro)……

Ha avuto trapianti? ☐ Si ☐ No Indichi il tipo di trapianto che ha avuto
……..

Soffre o ha sofferto di **depressione** (specificare se diagnosi clinica)? ……..

……

Soffre o ha sofferto di **mal di schiena** (episodi di più 1 giorno) nella zona **lombare o cervicale**?
SPECIFICARE……

……

Quanti episodi di **mal di schiena nella zona lombare** (lombalgia) ha avuto nella sua vita? (durati **almeno 1 giorno**)……

A che **età ha avuto il primo episodio di lombalgia**?……

Quante **ore al giorno** è esposto a **vibrazioni** ossia **viaggi in macchina, treno**, ecc……..

Fa **uso di farmaci** (compresa pillola contraccettiva o terapie ormonali, finasteride, ecc.) in maniera continuativa ? ☐ Si ☐ No

Farmaco utilizzato	Dosaggio	Periodo Da ……..a……..	Motivo

FOTOTIPO segnare con una croce su tipo di capelli, occhi, pelle, reazione al sole:

Tipo di pelle	I	II	III	IV	V	VI
Capelli	rossi, biondi	Rossi, biondi, castano chiaro	Castano, biondo scuro	Castano scuro	Castano scuro	Neri
Occhi	Blu, grigi, verdi	Blu, grigi, verdi, nocciola	marroni, blu, grigi, verdi, nocciola	Marroni	marroni	marroni
Pelle	Molto pallida, rossastra	Pallida	bianca, leggermente scura	Leggermente scura	Leggermente scura	nera
Reazione al sole	Si scotta facilmente, non si abbronza mai	Si scotta facilmente, si abbronza molto poco	A volte si scotta, si abbronza gradualmente	Si abbronza facilmente	Si abbronza facilmente e diventa scura	Si abbronza facilmente e diventa molto scura

Quanti **nevi** ha in tutto il corpo? Più di 50 ❑ meno di 50 ❑

Quando si espone **al sole si abbronza** ? Molto ❑ Mediamente ❑

Poco ❑ Per nulla ❑

Quando si è esposto al sole durante **l'ultimo anno** si è scottato?

❑ mai ❑ 1–2 volte ❑ 3–5 volte ❑ 6–10 volte ❑ >10 volte

Quando si è esposto al sole **nella sua vita** si è scottato?

❑ mai ❑ 1–2 volte ❑ 3–5 volte ❑ 6–10 volte ❑ >10 volte

D'estate per **quanti giorni** in media si espone al sole **in costume** ? .....
.....

D'estate per **quante ore** in media al giorno si espone al sole **in costume** ? .....
.....

In genere quando si espone al sole lo fa sotto **l'ombrellone o no** ?

SPECIFICARE……

Ricorda di avere avuto **importanti scottature dopo l'esposizione solare** durante l'infanzia e l'adolescenza ?

☐ Si (a che età……., quante volte ……..) ☐ No

Ricorda di avere avuto **importanti scottature** non dovute al sole come **acqua bollente, fuoco o altro** durante l'infanzia e l'adolescenza ?

☐ Si (a che età……., quante volte ……..) ☐ No

Fa uso o ha fatto uso di **lampade abbronzanti**? ☐ Si ☐ No

Se si mediamente quante lampade fa in un anno ☐ 1- 10 ☐ più di 10

Se si mediamente quanti minuti dura l'esposizione? ☐ ……..

Da quanti anni (o per quanti anni) ha fatto uso di lampade abbronzanti……

Da anno……..a anno……..

Esegue frequentemente **attività o sport all'aria aperta** ? ☐ Si ☐ No

SPECIFICARE **TIPO DI SPORT** E DURATA ESPOSIZIONE LUCE SOLARE

……

. ……..

……

Quando fa sport all'aperto come è vestito?.....

……

Ha dei tatuaggi? ☐ **NO** ☐ **SI**

Quanti tatuaggi? …… Circa quanto estesi ……

Colore/i tatuaggi ……..

Quando e dove sono stati eseguiti i tatuaggi? ……

Ha avuto **tumori della pelle**? ❑ Si ❑ No

Se si, SPECIFICARE tipo tumore, a che età è stato diagnosticato, parte del corpo, come trattato

……

……

……

Ha avuto **tumori** (**ma non della pelle**)? ❑ Si ❑ No

Se si, SPECIFICARE tipo tumore, a che età è stato diagnosticato, parte del corpo, come trattato

……

……

……

In **famiglia** qualcuno ha avuto un **melanoma**? ❑ Si ❑ No

Se si chi tra i suoi parenti ha sviluppato un melanoma? ……

……

……

Lei ha attualmente (o ha avuto) altre malattie dermatologiche come **psoriasi, vitiligine**, eczema, ecc?……

……

……

Tipo di psoriasi o vitiligine……

A che **età** è insorta la psoriasi o vitiligine ……..

In **famiglia** qualcuno ha avuto la **psoriasi**? ❑ Si ❑ No

Se si, chi tra i suoi parenti ha sviluppato la psoriasi? ……

……

In **famiglia** qualcuno ha avuto la **vitiligine** ? ❑ Si ❑ No

Se si, chi tra i suoi parenti ha sviluppato la vitiligine? ……..

……

In **famiglia** qualcuno ha avuto **Tumore al Pancreas** ? ❑ Si ❑ No

Se si, chi tra i suoi parenti ha avuto tumore al pancreas (a che età circa)?

……

In **famiglia** qualcuno ha avuto **Tumore al Cervello** ? ❑ Si ❑ No

Se si, chi tra i suoi parenti ha avuto tumore al cervello (a che età circa)?

……

……

In **famiglia** qualcuno ha avuto **Tumori diversi ad esempio tumore della prostata, della mammella, polmone, leucemie, ecc.** ? ❑ Si ❑ No

Se si, specificare tipo di tumore e chi tra i suoi parenti ha avuto tumori (a che età circa)?

……

……Soffre di **morbo di Parkinson**? ❑ Si ❑ No

Da quanti anni?……...

In famiglia qualcuno soffre o ha sofferto di **morbo di Parkinson**?……

……..

Data…… Firma ……...

Autorizzo il trattamento dei dati personali ai sensi della Legge 675/96.

Cancer Biol Med 2017. doi: 10.20892/j.issn.2095-3941.2017.0064

ORIGINAL ARTICLE

BsmI (rs1544410) and FokI (rs2228570) vitamin D receptor polymorphisms, smoking, and body mass index as risk factors of cutaneous malignant melanoma in northeast Italy

Sabina Cauci[1], Vincenzo Maione[2], Cinzia Buligan[1,3], Martina Linussio[1], Diego Serraino[4], Giuseppe Stinco[1,3]

[1]Department of Medicine, University of Udine, Udine 33100, Italy; [2]Dermatology Clinic University Hospital of Brescia, Brescia 25123, Italy; [3]Dermatology Clinic, University Hospital of Udine, Udine 33100, Italy; [4]Unit of Cancer Epidemiology, CRO Aviano National Cancer Institute, Aviano 33081, Italy

ABSTRACT

Objective: To investigate whether vitamin D receptor gene (*VDR*) BsmI-rs1544410 and FokI-rs2228570 polymorphisms, smoking duration, and body mass index (BMI) are risk factors for cutaneous melanoma, especially metastatic melanoma.

Methods: We studied 120 cutaneous melanoma cases [68 stage I and II non-metastatic melanoma (NMetM) patients, plus 52 Stage III and IV metastatic melanoma (MetM) patients], and 120 matching healthy controls from northeast Italy. *VDR* polymorphisms were measured by restriction fragment length polymorphism analysis. Absence or presence of BsmI and FokI restriction sites was denoted by "B" and "F" or by "b" and "f," respectively.

Results: *VDR*-BsmI bb genotype was more frequent among MetM (32.7%) than among NMetM cases (13.2%), with odds ratio (OR)=3.18. Comparison of all melanoma patients *vs* healthy controls showed that the following biomarkers were at risk: ≥20 years of smoking (OR=2.43); ≥20 years of smoking combined with bb (OR=4.78), Bb+bb (OR=2.30), Ff (OR=3.04), and Ff+ff (OR=3.08); obesity (BMI>30 kg/m^2) alone (OR=3.54); and obesity combined with Bb+bb (OR=3.52), Ff (OR=4.78), and Ff+ff (OR=6.56). Comparison of MetM *vs* NMetM patients revealed that the following biomarkers were at risk: ≥20 years of smoking (OR=2.39), ≥20 years of smoking combined with bb (OR=5.13), Bb+bb (OR=3.07), and Ff+ff (OR=2.66); and obesity combined with Bb+bb (OR=5.27), Ff (OR=6.28), and Ff+ff (OR=9.18). Triple combination of ≥20 years of smoking, obesity, and Bb+bb yielded OR=9.65 for melanoma patients *vs* healthy controls and OR=12.2 for MetM *vs*. NMetM patients.

Conclusions: Risk factors for cutaneous MetM include two *VDR* polymorphisms combined with smoking duration and obesity. Results suggest gene-environment implications in melanoma susceptibility and severity. Future studies in larger cohorts and in subjects with different genetic background are warranted to extend our findings.

KEYWORDS Vitamin D receptor; VDR polymorphism; cutaneous melanoma; metastatic melanoma; smoking; body mass index; obesity; skin cancer

Introduction

Melanoma continually presents increased incidence in all developed countries, particularly affecting fair-skinned individuals[1-3]. Malignant melanoma more frequently occurs in northern than in southern European countries[3]. Melanoma more frequently affects both sexes in Switzerland (European age standardized incidence rate 25.8/100,000/year) and Slovenia (20.6/100,000/year) than in Italy (13.4/100,000/year)[4]. Recent data in Italy[5] indicated a more than doubled prevalence of melanoma in northern than southern Italy, with central Italy presenting an intermediate value. Specifically, high incidence rates were recorded in Friuli-Venezia Giulia (FVG) region (19.6/100,000/year in men; 16.4/100,000/year in women) in northeast Italy[6]. This finding implies necessity for conducting geographically detailed studies regarding melanoma risk factors[7]. In the present study, we focused on inhabitants of the FVG region.

Critical environmental risk factors for melanoma include exposure to ultraviolet (UV) radiation, especially intermittent sun exposure and sunburns[8,9]. However, chronic and continuous UV ray exposure may yield protective effects[9,10] at least in part by activating synthesis of vitamin D, whose action is mediated by nuclear vitamin D receptor (VDR). Vitamin D-activated VDR may in turn up- or down-regulate several hundreds of genes by binding to vitamin D

Correspondence to: Sabina Cauci
E-mail: sabina.cauci@uniud.it
Received May 19, 2017; accepted June 30, 2017.
Available at www.cancerbiomed.org

responsive elements (VDREs), thus affecting several biological activities, such as calcium metabolism, immunity, detoxification, oxidative stress, cell proliferation, and differentiation[9-12]. Increasing evidence showed that vitamin D reduces risk of numerous types of cancer[12]. Thus, vitamin D endocrine system in studies concerning melanoma gained increasing attention[10,13,14]. Current studies and meta-analyses evaluated the role of the VDR gene (*VDR*) polymorphisms[12-22]. Nonetheless, *VDR* polymorphisms' roles still require further study[12,14,21].

The role of smoking in melanoma piqued interest of researchers[23,24]. Smoking is considered a risk factor for malignancies[25]. Paradoxically, several studies discovered inverse associations between smoking exposure and melanoma after controlling for potential confounding variables[24,26,27]. However, such protective effects are weak or insignificant[28,29]. Other studies did not confirm such association[30,31] or demonstrated tendencies toward smoking-related increased risks[10,32]. Thus, pathophysiological pathways underlying the relationship of smoking and melanoma currently poses a challenge in melanoma research[23,24].

Some studies on melanoma aimed to determine the role of body mass index (BMI) in occurrence of the disease[9,10,33-35]. However, limited research discusses combination of this biomarker with genetic traits.

Development in understanding of melanoma risk factors, genomics, and molecular pathogenesis may drive advances in precision medicine applied to melanoma[2,13,14].

Human *VDR* gene is located in chromosome 12q12-q14 and comprises 11 exons and 11 introns[18-22]. Most clinical studies that explored association of *VDR* polymorphisms with diseases[12,15,18,22] focused on two VDR single-nucleotide polymorphisms (SNPs), namely, BsmI-rs1544410 G>A located in intron 8 and FokI-rs2228570 C>T located in exon 2. These two polymorphisms show no linkage disequilibrium (LD)[18,22].

We explored *VDR* BsmI-rs1544410 and FokI-rs2228570 SNPs separately, and their association with lifestyle factors, particularly smoking duration and BMI of patients with cutaneous malignant melanomas, specifically those with metastatic melanoma (MetM) *vs.* non-metastatic melanoma (NMetM) and *vs.* healthy controls.

Patients and methods

Population

Enrollment and clinical visits of all study participants were performed at Dermatology Clinic, University Hospital of Udine. Diagnostic procedures were carried out according to routine protocols. The Udine Institutional Ethical Committee approved the study protocol, which was conducted according to the Declaration of Helsinki. All participants were alive during enrollment in the study and signed a written informed consent.

Using a case-control design, the study consecutively enrolled 120 (65 males and 55 females, age range of 31–84 years) unrelated patients (hospitalized or outpatients) with documented cutaneous melanoma diagnosis and 120 (65 males and 55 females, age range of 31–84 years) asymptomatic healthy controls, which were matched for gender, ancestry, and age with melanoma cases. Inclusion criteria for both melanoma cases and healthy controls were as follows: resident in FVG region, at least two grandparents born in FVG region (or Austro-Hungarian territory before World War I), and two grandparents, at the most, with central or southern Italian ancestry. Exclusion criteria for controls included the following: any kind of lifelong malignant or benign tumor, first-grade relatives with history of melanoma, and major chronic diseases, such as autoimmune diseases, type 1 diabetes, and thyroid diseases.

Melanoma was diagnosed using immunohistological findings obtained after surgical excision of nevi with clinical and dermoscopic characteristics suggesting presence of malignancy. Classification of melanoma stages was performed by clinical/histological/radiological findings, as described in final version of 2009 AJCC[36]. Inclusion criteria for case patients comprised cutaneous melanomas that were more severe than *in situ* only and with a Clark-grade invasion over I. For patients with multiple melanomas, the major melanoma characteristics were accounted for in study analyses according to histological assessment of major primary tumor (T) grading.

Each participant answered a questionnaire, which was used to collect data on demographic characteristics, medical and family history of melanoma, smoking habits, alimentary habits, and history of sunburns. Phototype was assessed by Fitzpatrick criteria[37]. BMI was determined by weight (kg) divided by squared height (m^2); BMI>30 kg/m^2 was considered as an indicator of obesity.

Genetic analysis of *VDR* polymorphisms

VDR-BsmI G>A and *VDR*-FokI C>T polymorphisms were determined, as described in Refs.[38,39], after extraction of genomic DNA from ethylenediaminetetraacetic-acid-treated venous blood samples[40]. Genotypes were designated according to absence/presence of the BsmI or FokI enzyme

restriction site by a capital letter B allele, or F allele for absence, and by a lowercase letter b allele, or f allele for presence, respectively[41]. FokI and BsmI polymorphisms of *VDR* were studied using previously tested primers[38-40], which were used to amplify appropriate DNA fragments. The following primers were specifically used: FokI-forward (5′-AGC TGG CCC TGG CAC TGA CTC TGC TCT-3′) and FokI-reverse (5′-ATG GAA ACA CCT TGC TTC TTC TCC CTC-3′); BsmI-forward (5′-CAA CCA AGA CTA CAA GTA CCG CGT CAG TGA-3′) and BsmI-reverse (5′-AAC CAG CGG GAA GAG GTC AAG GG-3′) primers. FokI enzyme (Euroclone, Milano, Italy) digestion of amplified 265 bp DNA fragment resulted in two 196 and 69 bp fragments in the presence of f allele[40]. To analyze BsmI polymorphism, the resulting amplified 825 bp fragment was digested with BsmI restriction enzyme (Euroclone, Milano, Italy), generating two fragments of 650 and 175 bp in the presence of b allele[39].

Statistical analysis

Continuous variables were expressed as mean ± standard deviation, and Mann-Whitney *U* test was performed for comparison. Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated for categorical variables, and *P* values for two-sided Pearson's Chi-squared or Fisher's exact test were reported as appropriate. Logistic regression was used to evaluate effects of confounders by obtaining adjusted ORs and CIs. Five different combinations of confounders were tested. Adjusted analysis included conventional risk factors: (1) gender and age; (2) gender, age, phototype 1+2, total number of body nevi>50, and number of lifelong sunburns>10. To compare MetM and NMetM, adjusted analyses included indicators that resulted in risk of metastasis development: (3) trunk location, Breslow's thickness, ulceration, mitosis>1, absence of tumor-infiltrating lymphocytes (TILs), and epithelioid variant; (4) ≥20 years of smoking; and (5) BMI>30 kg/m^2 (i.e., obesity). Adjusted analysis of type 3 confounders involved factors associated (according to our findings) with ≥20 years of smoking. These factors included TIL absence, ulceration and obesity. Thus, to avoid overcorrection, combined categorical variables, including smoking and obesity, were not adjusted for type 3 confounders.

Tests for deviations from Hardy-Weinberg equilibrium (HWE) were separately performed using chi-square distribution for each SNP[39,40]. LD between SNPs was determined as described by Colombini et al.[39]

A two-sided value of $P<0.05$ was considered significant, and $P\leq0.10$ indicates tendency to be significant. Statistical software SPSS for Windows (SPSS Inc., Chicago, IL, USA) was used.

Results

All 240 (120 cutaneous melanoma patients + 120 healthy controls) study subjects were Italian white residents in the FVG region.

Primary clinical characteristics of melanoma patients

As reported in **Table 1**, we examined in detail differences between MetM and NMetM patients to also identify appropriate variables to be included as confounders in subsequent multivariate analyses. Frequency of young (<40 years old) or old (≥60 years old) melanoma patients at study enrolment did not differ between MetM and NMetM groups (mean age comparisons reported in **Table 2**). Mean age at melanoma diagnosis reached 53.1±13.26 years. Mean time for melanoma diagnosis totaled 6.5±3.58 years and did not differ between MetM and NMetM patients.

The majority of 68 NMetM patients were in stage I (70.6%), whereas the majority of 52 MetM patients were in stage III (65.4%). Location in the trunk (OR=0.35) and superficial spreading (OR=0.31) showed protective effects for MetM patients *vs*. NMetM patients. Mean Breslow's thickness doubled in MetM cases *vs*. NMetM cases (2.8±1.74 *vs*. 1.4±1.34 mm, $P<0.001$). Specifically, a Breslow's thickness ≤0.75 mm had protective effects (OR=0.06), whereas thickness ≥4.01 mm was risky (OR=9.90) for MetM *vs*. NMetM cases. Some biomarkers were more frequently observed in MetM than in NMetM patients. These biomarkers included Clark IV invasion (OR=4.38), ulceration (OR=3.79), mitosis >1 (OR=3.77), TIL absence (OR=2.20), and epithelioid variant (OR=2.98).

Obesity and smoking history

By comparing obese and non-obese melanoma patients, we observed that non-brisk TIL cases were less frequent in obese (1/16, 6.25%) than in non-obese (40/104, 38.5%) patients, with OR=0.11, 95% CI=0.01–0.84, and $P=0.011$. By contrast, TIL absence was more frequent in obese (10/16, 62.5%) than in non-obese (31/104, 29.8%) melanoma patients, resulting in OR=3.92, 95% CI=1.31–11.7, and $P=0.010$.

Similar findings were observed by comparing melanoma patients who smoked ≥20 years *vs*. the remaining melanoma patients. Frequency of non-brisk TIL cases was lower in ≥20-year smokers (7/36, 19.4%) than other melanoma patients (34/84, 40.5%), yielding OR=0.35, 95% CI=0.14–0.90, and $P=0.026$. By contrast, TIL absence was more frequent in ≥20-

Table 1 Clinical characteristics of 120 consecutively enrolled melanoma patients and comparison between the two subgroups of 52 MetM and 68 NMetM patients

Characteristics	All melanoma patients (*n*=120)	MetM (*n*=52)	NMetM (*n*=68)	OR (CI) (MetM *vs*. NMetM)	*P* (MetM *vs*. NMetM)
Age, years, *n* (%)					
<40	9 (7.5)	3 (5.8)	6 (8.8)	0.63 (0.15–2.66)	0.730
≥ 60	60 (50.0)	27 (51.9)	33 (48.5)	1.14 (0.56–2.36)	0.713
Age at melanoma diagnosis (years, mean±SD)	53.1±13.26	53.9±13.01	52.5±13.51	–	0.569[a]
Time from melanoma diagnosis (years, mean±SD)	6.5±3.58	6.3±4.13	6.6±3.08	–	0.344[a]
Stage, *n* (%)					
I	48 (40.0)	0 (–)	48 (70.6)	–[b]	–[b]
II	20 (16.7)	0 (–)	20 (29.4)	–[b]	–[b]
III	34 (28.3)	34 (65.4)	0 (–)	–[b]	–[b]
IV	18 (15.0)	18 (34.6)	0 (–)	–[b]	–[b]
Trunk, *n* (%)	68 (56.7)	22 (42.3)	46 (67.6)	**0.35 (0.17–0.74)**	**0.006**
Upper limb, *n* (%)	8 (6.7)	2 (3.8)	6 (8.8)	0.41 (0.08–2.14)	0.463
Lower limb, *n* (%)	26 (21.7)	15 (28.8)	11 (16.2)	2.10 (0.87–5.07)	0.095^
Hands/feet, *n* (%)	8 (6.7)	6 (11.5)	2 (2.9)	4.30 (0.83–22.3)	0.076^
Head/neck, *n* (%)	10 (8.3)	7 (13.5)	3 (4.4)	3.37 (0.83–13.7)	0.099^
Superficial spreading, *n* (%)	56 (46.7)	16 (30.8)	40 (58.8)	**0.31 (0.14–0.67)**	**0.002**
Nodular, *n* (%)	47 (39.2)	25 (48.1)	22 (32.4)	1.94 (0.92–4.07)	0.080^
Acral lentiginous, *n* (%)	5 (4.2)	4 (7.7)	1 (1.5)	5.58 (0.60–51.5)	0.165
Lentigo maligna, *n* (%)	2 (1.7)	1 (1.9)	1 (1.5)	1.31 (0.08–21.5)	1.000
Spitzoide, *n* (%)	5 (4.2)	2 (3.8)	3 (4.4)	0.87 (0.14–5.38)	1.000
Others, *n* (%)	8 (6.7)	6 (11.5)	2 (2.9)	4.30 (0.83–22.3)	0.076^
Breslow thickness (mm, mean±SD)	2.0±1.66	**2.8±1.74**	**1.4±1.34**	–	**<0.001[a]**
Breslow thickness ≤0.75 mm, *n* (%)	28 (23.3)	2 (3.8)	26 (38.2)	**0.06 (0.01–0.29)**	**<0.001**
Breslow thickness ≥4.01 mm, *n* (%)	14 (11.7)	12 (23.1)	2 (2.9)	**9.90 (2.11–46.5)**	**0.001**
Clark II, *n* (%)	29 (24.2)	4 (7.7)	25 (36.8)	**0.14 (0.05–0.44)**	**<0.001**
Clark III, *n* (%)	20 (16.7)	4 (7.7)	16 (23.5)	**0.27 (0.08–0.87)**	**0.021**
Clark IV, *n* (%)	64 (53.3)	38 (73.1)	26 (38.2)	**4.38 (2.00–9.60)**	**<0.001**
Clark V, *n* (%)	5 (4.2)	4 (7.7)	1 (1.5)	5.58 (0.60–51.5)	0.165
Ulceration, *n* (%)	48 (40.0)	30 (57.7)	18 (26.5)	**3.79 (1.75–8.18)**	**0.001**
Mitosis >1, *n* (%)	81 (67.5)	43 (82.7)	38 (55.9)	**3.77 (1.59–8.94)**	**0.002**
Regression, *n* (%)	16 (13.3)	4 (7.7)	12 (17.6)	0.39 (0.12–1.28)	0.112
Brisk positive TILs[c], *n* (%)	37 (30.8)	12 (23.1)	25 (36.8)	0.52 (0.23–1.16)	0.108
Non-brisk TILs[c], *n* (%)	41 (34.2)	16 (30.8)	25 (36.8)	0.76 (0.35–1.65)	0.493

Continued

Continued

Characteristics	All melanoma patients (*n*=120)	MetM (*n*=52)	NMetM (*n*=68)	OR (CI) (MetM *vs*. NMetM)	*P* (MetM *vs*. NMetM)
TILs[c] absence, *n* (%)	41 (34.2)	23 (44.2)	18 (26.5)	**2.20 (1.02–4.75)**	**0.042**
Microsatellitosis, *n* (%)	4 (3.3)	3 (5.8)	1 (1.5)	4.10 (0.41–40.6)	0.315
Epithelioid variant, *n* (%)	30 (25.0)	19 (36.5)	11 (16.2)	**2.98 (1.27–7.03)**	**0.011**
Fusate variant, *n* (%)	12 (10.0)	7 (13.5)	5 (7.4)	1.96 (0.58–6.57)	0.269
Small cell variant, *n* (%)	2 (1.7)	0 (–)	2 (2.9)	–[b]	–[b]
More than 1 melanoma, *n* (%)	18 (15.0)	9 (17.3)	9 (13.2)	1.37 (0.50–3.74)	0.536
Additional non-melanoma skin cancer, *n* (%)	18 (15.0)	7 (13.5)	11 (16.2)	0.81 (0.29–2.25)	0.680
Additional non-skin cancer, *n* (%)	23 (19.2)	11 (21.2)	12 (17.6)	1.25 (0.50–3.12)	0.629
Melanoma familiarity	17 (14.2)	7 (13.5)	10 (14.7)	0.90 (0.32–2.57)	0.846

[a]Two-tailed Mann-Whitney *U*-test.[b]OR uncountable because one or two of the compared groups had zero subject. [c]TILs, tumor infiltrating lymphocytes. Significant differences were indicated in bold, tendencies were evidenced with superscript ^.

Table 2 Comparison of demographic characteristics of 120 melanoma patients and 120 healthy controls and comparison between the two subgroups of 52 MetM and 68 NMetM patients

Characteristics	All melanoma cases *n*=120	Healthy controls *n*=120	OR (CI) (melanomas *vs*. healthy controls)	*P* (melanomas *vs*. healthy controls)	MetM *n*=52	NMetM *n*=68	OR (CI) (MetM *vs*. NMetM)	*P* (MetM *vs*. NMetM)
Age, years, mean±SD	59.1±12.8	56.8±11.8	–	0.110[a]	60.2±12.1	58.3±13.4	–	0.503[a]
Age <50 years, *n* (%)	34 (28.3)	37 (30.8)	0.89 (0.51–1.54)	0.671	11 (21.1)	23 (33.8)	0.52 (0.23–1.21)	0.127
Males, *n* (%)	65 (54.2)	65 (54.2)	1.00 (0.60–1.66)	1.000	32 (61.5)	33 (48.5)	1.70 (0.81–3.53)	0.156
BMI, kg/m^2, mean±SD	**25.7±3.89**	**24.4±3.35**	–	**0.010[a]**	26.1±3.81	25.4±3.94	–	0.392[a]
BMI >25.0 kg/m^2, *n* (%)	63 (52.5)	52 (43.3)	1.44 (0.87–2.40)	0.155	30 (57.7)	33 (48.5)	1.45 (0.70–2.99)	0.319
BMI >30.0 kg/m^2, *n* (%)	16 (13.3)	5 (4.2)	**3.54 (1.25–10.0)**	**0.012**	9 (17.3)	7 (10.3)	1.82 (0.63–5.27)	0.263
Born in FVG region, *n* (%)	99 (82.5)	100 (83.3)	0.94 (0.48–1.85)	0.864	40 (76.9)	59 (86.8)	0.51 (0.20–1.32)	0.160
All 4 grand-parents born in FVG region, *n* (%)	85 (70.8)	83 (69.2)	1.08 (0.62–1.88)	0.778	35 (67.3)	50 (73.5)	0.74 (0.34–1.63)	0.457
Elementary school (5 study years), *n* (%)	17 (14.2)	8 (6.7)	2.31 (0.96–5.58)	0.057^	10 (19.2)	7 (10.3)	2.07 (0.73–5.89)	0.164
Low high-school (8 study years), *n* (%)	34 (28.3)	21 (17.5)	**1.86 (1.01–3.45)**	**0.046**	18 (34.6)	16 (23.5)	1.72 (0.77–3.83)	0.182
High-school (13 study years), *n* (%)	54 (45.0)	42 (35.0)	1.52 (0.90–2.55)	0.114	21 (40.4)	33 (48.5)	0.72 (0.35–1.49)	0.374
University level (laurea and/or master and/or PhD), *n* (%)	15 (12.5)	49 (40.8)	**0.21 (0.11–0.40)**	**<0.001**	3 (5.8)	12 (17.6)	0.29 (0.08–1.07)	0.051^

Continued

Continued

Characteristics	All melanoma cases *n*=120	Healthy controls *n*=120	OR (CI) (melanomas *vs*. healthy controls)	*P* (melanomas *vs*. healthy controls)	MetM *n*=52	NMetM *n*=68	OR (CI) (MetM *vs*. NMetM)	*P* (MetM *vs*. NMetM)
Phototype 1 or 2, *n* (%)	71 (59.2)	31 (25.8)	**4.16 (2.41–7.19)**	**<0.001**	34 (65.4)	37 (54.4)	1.58 (0.75–3.33)	0.226
Total Body Nevi >50, *n* (%)	63 (52.5)	41 (34.2)	**2.13 (1.27–3.58)**	**0.004**	28 (53.8)	35 (51.5)	1.10 (0.53–2.27)	0.796
Easy tanner, *n* (%)	4 (3.3)	22 (18.3)	**0.15 (0.05–0.46)**	**<0.001**	0 (–)	4 (5.9)	–[b]	–[b]
Medium tanner, *n* (%)	50 (41.7)	71 (59.2)	**0.49 (0.29–0.82)**	**0.007**	22 (42.3)	28 (41.2)	1.05 (0.50–2.18)	0.901
Low tanner, *n* (%)	58 (48.3)	26 (21.7)	**3.38 (1.93–5.94)**	**<0.001**	27 (51.9)	31 (45.6)	1.29 (0.62–2.66)	0.491
No tanner, *n* (%)	8 (6.7)	2 (1.7)	4.21 (0.88–20.3)	0.053^	3 (5.8)	5 (7.4)	0.77 (0.18–3.39)	1.000
Sunburns lifelong ≤5, *n* (%)	37 (30.8)	58 (48.3)	**0.48 (0.28–0.81)**	**0.006**	14 (26.9)	23 (33.8)	0.72 (0.33–1.59)	0.417
Sunburns lifelong 6–10, *n* (%)	21 (17.5)	28 (23.3)	0.70 (0.37–1.31)	0.262	12 (23.1)	9 (13.2)	1.97 (0.76–5.10)	0.160
Sunburns lifelong >10, *n* (%)	51 (42.5)	27 (22.5)	**2.55 (1.45–4.46)**	**0.001**	21 (40.4)	30 (44.1)	0.86 (0.41–1.78)	0.682
Present-smoker, *n* (%)	13 (10.8)	20 (16.7)	0.61 (0.29–1.29)	0.189	5 (9.6)	8 (11.8)	0.80 (0.24–2.60)	0.707
≥20 years smoking among present-smokers, *n* (%)	11 (84.6)	12 (60.0)	3.67 (0.64–21.1)	0.245	4 (80.0)	7 (87.5)	0.57 (0.03–11.8)	1.000
Years of smoking among present-smokers, mean±SD	27.7±13.7	23.4±13.5	–	0.386[a]	25.4±7.96	29.1±16.7	–	0.558[a]
N. cigarettes/day among present-smokers, mean±SD	14.4±9.99	9.83±6.26	–	0.234[a]	13.2±6.98	15.1±11.9	–	0.941[a]
Past-smoker, *n* (%)	46 (38.3)	28 (23.3)	**2.04 (1.17–3.58)**	**0.012**	23 (44.2)	23 (33.8)	1.55 (0.74–3.26)	0.245
≥20 years smoking among past-smokers, *n* (%)	25 (54.3)	6 (21.4)	**4.36 (1.49–12.8)**	**0.005**	17 (73.9)	8 (34.8)	**5.31 (1.50–18.8)**	**0.008**
Years of smoking among past-smokers, mean±SD	20.5±12.9	17.4±13.2	–	0.237[a]	**24.5±13.9**	**16.4±10.6**	–	**0.039[a]**
N. cigarettes/day among past-smokers, mean±SD	14.9±10.5	16.0±13.2	–	0.964[a]	13.8±9.85	16.0±11.3	–	0.424[a]
Years quitting smoking among past-smokers, mean±SD	20.2±12.3	20.6±14.5	–	0.978[a]	20.0±13.0	20.3±11.9	–	0.895[a]
Quitted smoking before first melanoma diagnosis among past-smokers, *n* (%)	39 (84.8)	–	–	–	19 (82.6)	20 (87.0)	0.71 (0.14–3.61)	1.000
Years of quitted smoking before first melanoma diagnosis, mean±SD	16.2±10.1	–	–	–	15.8±10.1	16.6±10.3	–	0.899
Ever-smoker, *n* (%)	59 (49.2)	48 (40.0)	1.45 (0.87–2.42)	0.153	28 (53.8)	31 (45.6)	1.39 (0.67–2.87)	0.370

Continued

Continued

Characteristics	All melanoma cases *n*=120	Healthy controls *n*=120	OR (CI) (melanomas *vs.* healthy controls)	*P* (melanomas *vs.* healthy controls)	MetM *n*=52	NMetM *n*=68	OR (CI) (MetM *vs.* NMetM)	*P* (MetM *vs.* NMetM)
≥20 years smoking among ever-smokers, n (%)	36 (61.0)	18 (37.5)	**2.61 (1.19–5.72)**	**0.016**	21 (75.0)	15 (48.4)	**3.20 (1.06–9.69)**	**0.036**
Years of smoking among ever-smokers, mean±SD	22.0±13.3	19.9±13.5	–	0.298[a]	24.7±12.9	19.7±13.4	–	0.086^[a]
N. cigarettes/day, among ever-smokers, mean±SD	14.8±10.3	13.4±11.2	–	0.335[a]	13.7±9.29	15.8±11.2	–	0.451[a]
≥20 years ever-smokers among all subjects, n (%)	36 (30.0)	18 (15.0)	**2.43 (1.29–4.58)**	**0.005**	21 (40.4)	15 (22.1)	**2.39 (1.08–5.31)**	**0.030**
Coffee drinker daily, n (%)	110 (91.7)	112 (93.3)	0.79 (0.30–2.06)	0.624	46 (88.5)	64 (94.1)	0.48 (0.13–1.79)	0.327
Coffee cups/day >3, n (%)	20 (16.7)	17 (14.2)	1.21 (0.60–2.45)	0.592	11 (21.1)	9 (13.2)	1.76 (0.67–4.62)	0.249

[a]Two-tailed Mann-Whitney *U*-test. [b]OR uncountable because one of the compared group had zero subject. Significant differences were indicated in bold, tendencies were evidenced with superscript ^.

year smokers (17/36, 47.2%) than in other melanoma patients (24/84, 28.6%), with OR=2.24, 95% CI=1.00–5.02, and *P*=0.048. By comparing ≥20-year smokers with the remaining melanoma patients, we detected significant findings for males (OR=4.45, 95% CI=1.81–10.9, *P*=0.001), stage III melanoma (OR=2.44, 95% CI=1.06–5.64, *P*=0.034), and ulceration (OR=2.50, 95% CI=1.12–5.56, *P*=0.023).

Comparison of demographic, behavioral, and environmental variables (Table 2)

Melanoma patients yielded higher mean BMI than healthy controls (*P*=0.010), and the number of obese subjects was over threefold higher (OR=3.54) among melanoma patients than among healthy controls.

Melanoma patients more frequently presented phototype 1+2 (OR=4.16), total number of body nevi>50 (OR=2.13), lifelong sunburns>10 (OR=2.55), and were more frequently low tanners (OR=3.38) than healthy controls. MetM patients did not differ from NMetM patients in terms of these characteristics.

Past smokers were twofold more frequent among melanoma patients than healthy controls. Among past smokers smoking for ≥20 years was considerably more frequent in melanoma patients (OR=4.36) than healthy controls and in MetM (OR=5.31) than NMetM patients. Past smokers with MetM showed higher average number of smoking years than NMetM patients (24.5±13.9 *vs.* 16.4±10.6 years; *P*=0.039). Among past smokers, melanoma patients quitted smoking for an average of 16.2±10.1 years before melanoma diagnosis, and differences were not observed between MetM and NMetM patients.

Twenty or more years of smoking among lifelong smokers and among all study subjects was a risk factor for melanoma patients *vs.* healthy controls (OR=2.61 and OR=2.43, respectively) and for MetM *vs.* NMetM patients (OR=3.20 and OR=2.39, respectively).

The majority of melanoma patients and healthy controls were daily coffee drinkers; no difference was noted among groups even when considering those who consumed over three cups of coffee per day.

Unadjusted comparisons of *VDR*-BsmI and *VDR*-FokI genotypes alone or combined with smoking and obesity (Table 3)

VDR-BsmI and *VDR*-FokI genotypes were in HWE in healthy controls and in melanoma patients. As expected, the two SNPs were not in LD.

Homozygous bb genotype was more frequent among MetM than among NMetM patients (OR=3.18). Intriguingly, bb frequency was lower in NMetM patients than in healthy controls (OR=0.40). Genotype bb combined with ≥20 years of smoking was more frequent among all melanoma patients than healthy controls (OR=4.78), in MetM than NMetM patients (OR=5.13), and in MetM patients than healthy controls (OR=9.18). The same profile was observed for Bb+bb (b allele carriers) plus ≥20 years of smoking. Carriers

Table 3 *VDR*-BsmI and *VDR*-FokI genotypes alone or combined with smoking duration and obesity compared between 120 melanoma cases and 120 healthy controls.

Single or combined variable	Melanoma cases (*n*=120), *n* (%)	Healthy controls (*n*=120), *n* (%)	OR (CI), *P* (Melanomas *vs*. healthy controls)	MetM (*n*=52), *n* (%)	NMetM (*n*=68), *n* (%)	OR (CI), *P* (MetM *vs*. NMetM)	OR (CI), *P* (MetM *vs*. healthy controls)	OR (CI), *P* (NMetM *vs*. healthy controls)
BB	30 (25.0)	31 (25.8)	0.96 (0.53–1.71), 0.882	11 (21.2)	19 (27.9)	0.69 (0.30–1.62), 0.395	0.77 (0.35–1.68), 0.512	1.11 (0.57–2.17), 0.753
Bb	64 (53.3)	56 (46.7)	1.31 (0.79–2.17), 0.302	24 (46.2)	40 (58.8)	0.60 (0.29–1.24), 0.168	0.98 (0.51–1.88), 0.951	1.63 (0.89–2.98), 0.109
bb	26 (21.7)	33 (27.5)	0.73 (0.40–1.32), 0.294	17 (32.7)	9 (13.2)	**3.18 (1.28–7.91), 0.010**	1.28 (0.63–2.59), 0.491	**0.40 (0.18–0.90), 0.024**
BB+Bb (B allele)	94 (78.3)	87 (72.5)	1.37 (0.76–2.48), 0.294	35 (67.3)	59 (86.8)	**0.31 (0.13–0.78), 0.010**	0.78 (0.39–1.58), 0.491	**2.49 (1.11–5.58), 0.024**
Bb+bb (b allele)	90 (75.0)	89 (74.2)	1.04 (0.58–1.87), 0.882	41 (78.8)	49 (72.1)	1.44 (0.62–3.38), 0.395	1.30 (0.59–2.83), 0.512	0.90 (0.46–1.75), 0.753
bb plus ≥20 years ever-smoking	9 (7.5)	2 (1.7)	**4.78 (1.01–22.6), 0.031**	7 (13.5)	2 (2.9)	**5.13 (1.02–25.8), 0.039**	**9.18 (1.84–45.8), 0.004**	1.79 (0.25–13.0), 0.621
Bb+bb plus ≥20 years ever-smoking	28 (23.3)	14 (11.7)	**2.30 (1.14–4.64), 0.017**	18 (34.6)	10 (14.7)	**3.07 (1.27–7.41), 0.011**	**4.01 (1.80–8.90), <0.001**	1.30 (0.55–3.12), 0.549
bb plus BMI >30 kg/m^2	4 (3.3)	0 (–)	–[a]	3 (5.8)	1 (1.5)	4.10 (0.41–40.6), 0.315	–[a]	–[a]
Bb+bb plus BMI >30 kg/m^2	13 (10.8)	4 (3.3)	**3.52 (1.11–11.1), 0.024**	8 (15.4)	5 (7.4)	2.29 (0.70–7.47), 0.161	**5.27 (1.51–18.4), 0.008**	2.30 (0.60–8.88), 0.288
Bb+bb plus ≥20 years ever-smoking, and plus BMI >30 kg/m^2	9 (7.5)	1 (0.8)	**9.65 (1.20–77.4), 0.010**	8 (15.4)	1 (1.5)	**12.2 (1.47–101), 0.010**	**21.6 (2.63–178), <0.001**	1.78 (0.11–28.9), 1.00
FF	47 (39.2)	54 (45.0)	0.79 (0.47–1.31), 0.360	17 (32.7)	30 (44.1)	0.61 (0.29–1.30), 0.204	0.59 (0.30–1.17), 0.132	0.96 (0.53–1.76), 0.907
Ff	60 (50.0)	50 (41.7)	1.40 (0.84–2.33), 0.195	29 (55.8)	31 (45.6)	1.50 (0.73–3.11), 0.269	1.76 (0.91–3.40), 0.088^	1.17 (0.64–2.14), 0.602
ff	13 (10.8)	16 (13.3)	0.79 (0.36–1.72), 0.552	6 (11.5)	7 (10.3)	1.14 (0.36–3.61), 0.828	0.85 (0.31–2.31), 0.746	0.75 (0.29–1.91), 0.541
FF+ff (F allele)	107 (89.2)	104 (86.7)	1.27 (0.58–2.76), 0.552	46 (88.5)	61 (89.7)	0.88 (0.28–2.79), 0.828	1.18 (0.43–3.21), 0.746	1.34 (0.52–3.44), 0.541
Ff+ff (f allele)	73 (60.8)	66 (55.0)	1.27 (0.76–2.12), 0.360	35 (67.3)	38 (55.9)	1.62 (0.77–3.45), 0.204	1.68 (0.85–3.33), 0.132	1.04 (0.57–1.89), 0.907

Continued

Continued

Single or combined variable	Melanoma cases (*n*=120), *n* (%)	Healthy controls (*n*=120), *n* (%)	OR (CI), *P* (Melanomas *vs.* healthy controls)	MetM (*n*=52), *n* (%)	NMetM (*n*=68), *n* (%)	OR (CI), *P* (MetM *vs.* NMetM)	OR (CI), *P* (MetM *vs.* healthy controls)	OR (CI), *P* (NMetM *vs.* healthy controls)
Ff plus ≥20 years ever-smoking	19 (15.8)	7 (5.8)	**3.04 (1.23–7.52), 0.013**	12 (23.1)	7 (10.3)	2.61 (0.95–7.21), 0.057^	**4.84 (1.78–13.2), 0.001**	1.85 (0.62–5.53), 0.263
Ff+ff plus ≥20 years ever-smoking	24 (20.0)	9 (7.5)	**3.08 (1.37–6.95), 0.005**	15 (28.8)	9 (13.2)	**2.66 (1.06–6.69), 0.034**	**5.00 (2.02–12.4), <0.001**	1.88 (0.71–4.99), 0.199
Ff plus BMI >30 kg/m^2	9 (7.5)	2 (1.7)	**4.78 (1.01–22.6), 0.031**	5 (9.6)	4 (5.9)	1.70 (0.43–6.68), 0.499	**6.28 (1.18–33.5), 0.027**	3.69 (0.66–20.7), 0.191
Ff+ff plus BMI >30 kg/m^2	12 (10.0)	2 (1.7)	**6.56 (1.43–30.0), 0.006**	7 (13.5)	5 (7.4)	1.96 (0.58–6.57), 0.269	**9.18 (1.84–45.8), 0.004**	4.68 (0.88–24.8), 0.101
Ff+ff plus ≥20 years ever-smoking, and plus BMI >30 kg/m^2	6 (5.0)	0 (–)	–[a]	6 (11.5)	0 (–)	–[a]	–[a]	–[a]

[a]OR uncountable because one or two of the compared groups had zero subject. Significant differences were indicated in bold, tendencies were evidenced with superscript ^.

of b allele (Bb+bb) who were obese showed increased risk for all melanomas (OR=3.52 for melanoma patients *vs.* healthy controls) and MetM (OR=5.27 for MetM *vs.* healthy controls). Notably, the combination of three parameters, i.e., Bb+bb genotype plus ≥20 years of smoking plus obesity yielded high ORs for all melanoma patients *vs.* healthy controls (OR=9.65), MetM *vs.* NMetM patients (OR=12.2), and MetM patients *vs.* healthy controls (OR=21.6).

As shown in **Table 3**, *VDR*-FokI genotype FF, Ff, and ff frequencies did not differ among groups. However, heterozygous Ff had a tendency to be more frequent among MetM patients than healthy controls (OR=1.76; *P*=0.088). Notably, Ff genotype combined with ≥20 years of smoking acted as risk factor for all melanoma patients (OR=3.04 for melanoma patients *vs.* healthy controls) and MetM patients (OR=4.84 for MetM patients *vs.* healthy controls). Carriers of f allele (i.e., Ff+ff) combined with ≥20 years of smoking posed risk for all melanoma patients (OR=3.08 for melanoma patients *vs.* healthy controls) and for MetM (OR=5.00 for MetM patients *vs.* healthy controls, and OR=2.66 for MetM *vs.* NMetM patients). Ff genotype combined with obesity exhibited OR=4.78 for all melanoma patients *vs.* healthy controls and OR=6.28 for MetM patients *vs.* healthy controls. Finally, obese carriers of Ff+ff presented an increased risk for all melanomas (OR=6.56 for all melanoma patients *vs.* healthy controls) and for MetM (OR=9.18 for MetM patients *vs.* healthy controls). Notably, only 6 out of 240 study subjects showed the triple combination of Ff+ff genotype, ≥20 years of smoking, and obesity, and they were all MetM patients.

Comparisons of *VDR*-BsmI and *VDR*-FokI genotypes, smoking, and obesity alone or their combinations (Table 4)

As shown in **Table 4**, by comparing all 120 melanoma cases *vs.* 120 healthy controls, four variables including the parameter ≥20 years of ever smoking among all subjects were significant after multivariate analysis of type 1 confounders (including gender and age): ≥20 years of smoking alone (OR=2.19), or plus Bb+bb (OR=2.08), plus Ff (OR=2.77), and plus Ff+ff (OR=2.86) genotype. However, all those differences became not significant adding more confounding factors by analysis of type 2 confounders. Multivariate analysis of type 2 confounders revealed that five variables, including obesity, were all risk factors for melanoma patients *vs.* healthy controls, and they were as follows: BMI>30 kg/m^2 alone (OR=5.28), or plus Bb+bb (OR=4.35), plus Ff (OR=6.91), and plus Ff+ff (OR=8.89) genotype, and triple combination of Bb+bb, ≥20 years of smoking, and obesity (OR=12.0).

Comparison of MetM patients *vs* healthy controls revealed

Table 4 Association of ≥20 years of smoking, obesity, *VDR*-BsmI genotype, and *VDR*-FokI genotype as single or combined with variables of melanoma.

Single or combined variable	Adjusted[1] OR (CI), *P* (All melanoma cases *vs*. healthy controls)	Adjusted[2] OR (CI), *P* (Melanomas *vs*. healthy controls)	Adjusted[1] OR (CI), *P* (MetM *vs*. healthy controls)	Adjusted[2] OR (CI), *P* (MetM *vs*. healthy controls)	Adjusted[1] OR (CI), *P* (NMetM *vs*. healthy controls)	Adjusted[2] OR (CI), *P* (NMetM *vs*. healthy controls)
≥20 years of ever-smoking	**2.19 (1.13–4.24), 0.020**	1.79 (0.85–3.74), 0.124	**3.16 (1.44–6.95), 0.004**	**2.46 (1.00–6.05), 0.050**	1.50 (0.69–3.28), 0.304	1.13 (0.47–2.73), 0.789
BMI >30 kg/m^2	**3.20 (1.12–9.14), 0.030**	**5.28 (1.73–16.1), 0.003**	**3.95 (1.22–12.8), 0.022**	**7.74 (2.02–29.6), 0.003**	2.51 (0.76–8.33), 0.132	**3.69 (1.04–13.1), 0.043**
BB	0.92 (0.51–1.66), 0.777	0.99 (0.52–1.91), 0.982	0.76 (0.34–1.68), 0.495	0.93 (0.39–2.25), 0.881	1.06 (0.54–2.08), 0.874	1.01 (0.48–2.12), 0.975
Bb	1.47 (0.87–2.49), 0.147	1.48 (0.83–2.63), 0.186	1.09 (0.56–2.12), 0.808	0.89 (0.41–1.92), 0.768	**1.88 (1.01–3.49), 0.045**	**2.15 (1.09–4.25), 0.028**
bb	0.66 (0.36–1.20), 0.171	0.61 (0.31–1.18), 0.140	1.15 (0.56–2.35), 0.705	1.21 (0.53–2.77), 0.644	**0.36 (0.16–0.81), 0.013**	**0.30 (0.12–0.75), 0.010**
BB+Bb (B allele)	1.52 (0.83–2.76), 0.171	1.65 (0.85–3.19), 0.140	0.87 (0.42–1.78), 0.705	0.82 (0.36–1.88), 0.644	**2.81 (1.24–6.36), 0.013**	**3.30 (1.34–8.15), 0.010**
Bb+bb (b allele)	1.09 (0.60–1.97), 0.777	1.01 (0.52–1.94), 0.982	1.32 (0.60–2.92), 0.495	1.07 (0.44–2.58), 0.881	0.95 (0.48–1.86), 0.874	0.99 (0.47–2.08), 0.975
bb plus ≥20 years of smoking	4.16 (0.86–20.2), 0.077^	3.24 (0.58–17.9), 0.179	**6.81 (1.27–36.6), 0.025**	**6.99 (1.08–45.2), 0.041**	1.68 (0.23–12.4), 0.612	0.98 (0.10–9.54), 0.987
Bb+bb plus ≥20 years of smoking	**2.08 (1.00–4.29), 0.049**	1.75 (0.78–3.92), 0.171	**3.29 (1.42–7.60), 0.005**	2.42 (0.93–6.28), 0.070^	1.19 (0.49–2.89), 0.702	0.94 (0.36–2.51), 0.910
bb plus BMI >30 kg/m^2	–[a]	–[a]	–[a]	–[a]	–[a]	–[a]
Bb+bb plus BMI >30 kg/m^2	**3.20 (1.00–10.3), 0.050**	**4.35 (1.28–14.8), 0.018**	**4.34 (1.22–15.5), 0.024**	**6.55 (1.60–26.9), 0.009**	2.26 (0.58–8.85), 0.242	2.81 (0.67–11.8), 0.158
Bb+bb plus ≥20 years of smoking, and plus BMI >30 kg/m^2	**8.61 (1.06–69.8), 0.044**	**12.0 (1.40–104), 0.024**	**17.4 (2.07–146), 0.008**	**22.7 (2.46–209), 0.006**	1.93 (0.12–32.1), 0.646	2.68 (0.14–49.8), 0.508
FF	0.80 (0.47–1.36), 0.413	0.90 (0.50–1.62), 0.732	0.59 (0.29–1.20), 0.145	0.60 (0.27–1.33), 0.206	0.97 (0.53–1.80), 0.935	1.12 (0.57–2.19), 0.749
Ff	1.30 (0.77–2.20), 0.317	1.21 (0.68–2.17), 0.516	1.70 (0.87–3.35), 0.122	1.59 (0.73–3.45), 0.243	1.11 (0.60–2.05), 0.728	1.03 (0.53–2.03), 0.923
ff	0.88 (0.39–1.98), 0.758	0.81 (0.33–1.96), 0.638	0.90 (0.32–2.54), 0.846	1.08 (0.34–3.41), 0.902	0.82 (0.31–2.14), 0.682	0.72 (0.25–2.03), 0.531
FF+Ff (F allele)	1.13 (0.51–2.55), 0.758	1.24 (0.51–3.01), 0.638	1.11 (0.39–3.12), 0.846	0.93 (0.29–2.95), 0.902	1.22 (0.47–3.21), 0.682	1.39 (0.49–3.96), 0.531
Ff+ff (f allele)	1.25 (0.73–2.12), 0.413	1.11 (0.62–1.99), 0.732	1.69 (0.83–3.43), 0.145	1.68 (0.75–3.74), 0.206	1.03 (0.55–1.89), 0.935	0.90 (0.46–1.75), 0.749
Ff plus ≥20 years of smoking	**2.77 (1.10–6.98), 0.031**	2.36 (0.84–6.58), 0.102	**4.02 (1.45–11.1), 0.007**	**3.69 (1.12–12.2), 0.032**	1.78 (0.59–5.39), 0.307	1.48 (0.42–5.23), 0.545
Ff+ff plus ≥20 years of smoking	**2.86 (1.24–6.60), 0.013**	2.30 (0.91–5.82), 0.080^	**4.21 (1.65–10.7), 0.003**	**3.92 (1.31–11.7), 0.014**	1.83 (0.68–4.93), 0.234	1.31 (0.42–4.11), 0.646
Ff plus BMI >30 kg/m^2	4.41 (0.92–21.1), 0.063^	**6.91 (1.35–35.4), 0.020**	**6.09 (1.11–33.4), 0.037**	**14.6 (2.16–98.3), 0.006**	3.61 (0.63–20.5), 0.148	4.39 (0.71–27.2), 0.112
Ff+ff plus BMI >30 kg/m^2	**6.09 (1.32–28.0), 0.020**	**8.89 (1.82–43.4), 0.007**	**8.50 (1.66–43.4), 0.010**	**17.8 (2.82–112), 0.002**	4.53 (0.85–24.3), 0.078^	5.63 (0.98–32.4), 0.053^

[1]Adjusted OR (CI) for gender, and age. [2]Adjusted OR for gender, age, phototype 1+2, total body nevi >50, and >10 lifelong sunburns. [a]OR uncountable because one of the compared group had zero subject. Significant differences were indicated in bold, tendencies were evidenced with superscript ^.

that combination of Bb+bb and ≥20 years of smoking was significant (OR=3.29) after adjustment of type 1 confounders, but became a tendency after adjustment of type 2 confounders. By contrast, eight other variables were significant after both multivariate analyses of types 1 and 2 confounders. Specifically, by analysis of type 2 confounders, the following significant findings were observed: ≥20 years of smoking alone (OR=2.46) or combined with bb (OR=6.99), Ff (OR=3.69), and Ff+ff (OR=3.92) genotype; and obesity alone (OR=7.74) or combined with Bb+bb (OR=6.55), Ff (OR=14.6), and Ff+ff (OR=17.8) genotype. Notably, triple combination of Bb+bb, ≥20 years of smoking, and obesity resulted in type 2-adjusted OR=22.7 for MetM patients *vs* healthy controls.

Adjusted comparisons of type 2 confounders among NMetM patients *vs* healthy controls revealed risk effects of obesity (OR=3.69), Bb (OR=2.15), and BB+Bb (OR=3.30) genotypes.

Comparisons of *VDR*-BsmI and *VDR*-FokI genotypes, smoking, and obesity alone or their combinations (Table 5)

Table 5 illustrates comparison of MetM *vs* NMetM patients by adjusted analyses of types 1 to 5 confounders. Smoking duration of ≥20 years is a significant risk factor for MetM *vs*. NMetM patients (OR=2.26, 95% of CI=1.00–5.10, *P*=0.050) after adjustment for obesity (type 5 confounder). However, this risk factor became a tendency after extensive adjustments (types 1 and 2 confounders). Notably, bb genotype showed consistent risky adjusted OR=3 for MetM *vs*. NMetM after multivariate analysis of types 1 to 5 confounders. Consequently, carriage of B allele (i.e., BB+Bb) resulted in protective effects with respect to MetM. Significant threefold increased risk for MetM *vs*. NMetM cases was observed for the combination of Bb+bb and ≥20 years of smoking after adjustments for types 1, 2, and 5 confounders. Finally, triple combination of Bb+bb, ≥20 years of smoking, and obesity showed high types 1 and 2-adjusted ORs (OR=10.7 and OR=11.8, respectively), thus attesting for gene-behavioral effects among MetM patients.

Discussion

Our study was carried out under the context of precision medicine approach for disease treatment and prevention, which considers individual variability in genes, environment, and lifestyles[42].

VDR-BsmI polymorphism

We observed similar general distribution of *VDR*-BsmI genotypes (BB 25.0%, Bb 53.3%, bb 21.7%) among all

Table 5 Association of ≥20 years of smoking, obesity, *VDR*-BsmI genotype, and *VDR*-FokI genotype as single or combined variables with MetM (*n*=52) *vs*. NMetM (*n*=68), as evaluated by adjusted[1,2,3,4,5] OR (CI)

Single or combined variable	Adjusted[1] OR (CI), *P* (MetM *vs*. NMetM)	Adjusted[2] OR (CI), *P* (MetM *vs*. NMetM)	Adjusted[3] OR (CI), *P* (MetM *vs*. NMetM)	Adjusted[4] OR (CI), *P* (MetM *vs*. NMetM)	Adjusted[5] OR (CI), *P* (MetM *vs*. NMetM)
≥20 years of smoking	2.14 (0.92–4.94), 0.075^	2.14 (0.91–5.04), 0.081^	–[a]	–[a]	**2.26 (1.00–5.10), 0.050**
BMI >30 kg/m^2	1.60 (0.54–4.75), 0.398	1.82 (0.58–5.77), 0.306	–[a]	1.45 (0.48–4.37), 0.511	–[a]
BB	0.77 (0.32–1.85), 0.563	0.85 (0.35–2.05), 0.712	0.96 (0.34–2.77), 0.945	0.71 (0.30–1.69), 0.439	0.71 (0.30–1.66), 0.429
Bb	0.52 (0.24–1.12), 0.094^	0.47 (0.21–1.02), 0.058^	0.45 (0.18–1.12), 0.086^	0.59 (0.28–1.24), 0.166	0.59 (0.28–1.23), 0.159
bb	**3.28 (1.30–8.23), 0.012**	**3.42 (1.34–8.76), 0.010**	**3.06 (1.05–8.88), 0.040**	**3.16 (1.25–7.99), 0.015**	**3.17 (1.27–7.91), 0.013**
BB+Bb (B allele)	**0.30 (0.12–0.77), 0.012**	**0.29 (0.11–0.75), 0.010**	**0.33 (0.11–0.95), 0.040**	**0.32 (0.12–0.80), 0.015**	**0.31 (0.13–0.79), 0.013**
Bb+bb (b allele)	1.29 (0.54–3.09), 0.563	1.18 (0.49–2.87), 0.712	1.04 (0.36–2.98), 0.945	1.41 (0.59–3.35), 0.439	1.14 (0.60–3.32), 0.429
bb and ≥20 years of smoking	4.38 (0.85–22.7), 0.078^	4.38 (0.82–23.3), 0.083^	–[a]	–[a]	4.73 (0.92–24.2), 0.062^

Continued

Continued

Single or combined variable	Adjusted[1] OR (CI), *P* (MetM *vs*. NMetM)	Adjusted[2] OR (CI), *P* (MetM *vs*. NMetM)	Adjusted[3] OR (CI), *P* (MetM *vs*. NMetM)	Adjusted[4] OR (CI), *P* (MetM *vs*. NMetM)	Adjusted[5] OR (CI), *P* (MetM *vs*. NMetM)
Bb+bb and ≥20 years of smoking	**2.79 (1.07–7.22), 0.035**	**3.02 (1.13–8.09), 0.027**	_[a]	_[a]	**2.92 (1.16–7.32), 0.023**
bb plus BMI >30 kg/m^2	3.22 (0.32–32.8), 0.323	3.37 (0.31–37.1), 0.320	_[a]	2.92 (0.28–30.4), 0.371	_[a]
Bb+bb plus BMI >30 kg/m^2	1.94 (0.57–6.55), 0.285	2.10 (0.59–7.42), 0.250	_[a]	1.66 (0.48–5.76), 0.426	_[a]
Bb+bb plus ≥20 years of smoking, and plus BMI >30 kg/m^2	**10.7 (1.26–90.7), 0.030**	**11.8 (1.33–105), 0.027**	_[a]	_[a]	_[a]
FF	0.65 (0.30–1.39), 0.269	0.68 (0.31–1.49), 0.341	0.65 (0.26–1.61), 0.353	0.64 (0.30–1.39), 0.261	0.64 (0.30–1.37), 0.249
Ff	1.43 (0.68–2.99), 0.342	1.46 (0.68–3.16), 0.332	1.01 (0.42–2.46), 0.974	1.48 (0.71–3.11), 0.296	1.48 (0.71–3.07), 0.291
ff	1.14 (0.35–3.72), 0.830	0.98 (0.28–3.36), 0.970	3.32 (0.73–15.1), 0.120	1.05 (0.32–3.43), 0.937	1.07 (0.33–3.43), 0.915
FF+Ff (F allele)	0.88 (0.27–2.87), 0.830	1.02 (0.30–3.53), 0.970	0.30 (0.07–1.37), 0.120	0.95 (0.29–3.11), 0.937	0.94 (0.29–3.02), 0.915
Ff+ff (f allele)	1.54 (0.72–3.29), 0.269	1.46 (0.67–3.18), 0.341	1.54 (0.62–3.82), 0.353	1.55 (0.72–3.34), 0.261	1.56 (0.73–3.33), 0.249
Ff plus ≥20 years of smoking	2.27 (0.79–6.48), 0.126	2.31 (0.79–6.72), 0.126	_[a]	_[a]	2.51 (0.91–6.97), 0.077^
Ff+ff plus ≥20 years of smoking	2.34 (0.89–6.13), 0.083^	2.34 (0.87–6.28), 0.093^	_[a]	_[a]	2.51 (0.99–6.39), 0.054^
Ff plus BMI >30 kg/m^2	1.46 (0.36–5.89), 0.593	1.59 (0.38–6.65), 0.521	_[a]	1.52 (0.37–6.16), 0.559	_[a]
Ff+ff plus BMI >30 kg/m^2	1.73 (0.51–5.92), 0.382	1.81 (0.51–6.44), 0.357	_[a]	1.67 (0.48–5.77), 0.421	_[a]

[1]Adjusted OR (CI) for gender, and age. [2]Adjusted OR for gender, age, phototype 1+2, total body nevi >50, and >10 lifelong sunburns.[3]Adjusted OR for trunk location, Breslow thickness, ulceration, mitosis >1, TILs absence, and epithelioid variant.[4]Adjusted OR for ≥20 years of smoking.[5]Adjusted OR for obesity (BMI >30 kg/m^2).[a]Non calculated to avoid over-correction as described in "Methods". Significant differences were indicated in bold, tendencies were evidenced with superscript ^.

melanoma patients and healthy controls (BB 25.8%, Bb 46.7%, bb 27.5%), showing agreement with other case-control investigations[16,20]. Notably, a threefold higher frequency of bb genotype was observed in MetM (32.7%) compared with NMetM cases (13.2%); this value ranged from significant crude OR=3.18 to adjusted ORs ranging from 3.06 to 3.42 after considering several confounders. We observed that B carriers (BB+Bb) were at reduced risk comparing MetM *vs*. NMetM cases. However, B carriers were at increased risk when comparing NMetM *vs*. healthy controls. Paradoxically, by comparison with healthy controls, carriage of bb genotype posed risk to MetM, but was protective for NMetM cases. In this study, distributions of genotypes in melanoma patients were similar with respect to Bb frequencies of those observed in central Italy by Santonocito et al.[15] in 101 melanoma patients (BB 9.9%, Bb 53.5%, bb 36.6%). The study indicated increased frequencies of Bb and bb genotypes in melanoma patients compared with healthy controls (BB 23.8%, Bb 50.5%, bb 25.7%) and demonstrated an association between *VDR*-BsmI bb genotype and increased Breslow's thickness[15], a parameter that is consistently associated with metastasis and poor prognosis[9]. A meta-analysis[17] showed that BsmI B allele is associated with reduced melanoma risk with OR=0.81 and 95% CI=0.72–0.92. A large-scale study of incidence of multiple primary melanoma revealed distribution of *VDR*-BsmI, with values of BB 18.9%, Bb 46.8%, and bb 34.2%, among patients with multiple primary melanomas and BB

15.3%, Bb 47.7%, and bb 37.0% among patients with single primary melanoma[19]. A recent meta-analysis[22] reported a 15% decrease in melanoma risk (pooled OR=0.85, 95% CI=0.76–0.94) for individuals with BB or Bb genotype compared with subjects featuring bb genotype.

In our study, bb genotype combined with ≥20 years of smoking yielded adjusted OR=7 for MetM patients *vs* healthy controls. Bb+bb (i.e., b allele) genotype combined with obesity showed adjusted ORs from 4 to 7 for MetM patients *vs* healthy controls.

Functional effect of *VDR*-BsmI polymorphism remains unclear[39,41,43]. This SNP is located in an intron sequence at the 3' end of *VDR* gene. Thus, *VDR*-BsmI polymorphism cannot directly change the protein sequence of the VDR receptor. Some studies suggested that this SNP can influence *VDR*-mRNA expression, thereby affecting its stability[43]. BsmI site may be in LD with other truly relevant SNPs in *VDR* or other genes[14,15,44].

VDR-FokI polymorphism

In our study, *VDR*-FokI genotypes (FF 39.2%, Ff 50.0%, and ff 10.8% in melanoma cases *vs*. FF 45.0%, Ff 41.7%, and ff 13.3% in healthy controls) were not associated with melanoma, and this result agrees with results of a recent meta-analysis[21]. However, we noted an increased risk for heterozygous Ff carriers when we compared MetM *vs*. NMetM cases. A Serbian study showed that compared with ff genotype, Ff and FF were associated with increased melanoma risk (OR=3.03, P=0.003; OR=9.28, P<0.001, respectively)[45]. In general, inconsistent findings were reported for association of *VDR*-FokI polymorphism with melanoma[19,46]. In one meta-analysis[20], FokI polymorphism was associated with an overall significantly increased risk of skin cancer (Ff *vs*. FF: OR=1.20, 95% CI=1.01–1.44; ff *vs*. FF: OR=1.41, 95% CI=1.08–1.84; Ff+ff *vs*. FF: OR=1.26, 95% CI=1.04–1.53). Another meta-analysis[22] claimed that f allele carriers showed an 18% (pooled OR=1.18, 95% CI=1.07–1.29) increased risk for melanoma compared with FF homozygotes. Notably, in our study, Ff+ff (f allele carriers), when combined with ≥20 years of smoking or with obesity, exhibited adjusted OR=4 and ORs from 8 to 18, respectively, for MetM patients *vs*. healthy controls. The f allele codes for a 427 amino acids long VDR protein, and it is considered less effective than the protein receptor coded by F allele (424 amino acids long)[40,41,43].

Smoking

Our study highlighted the crucial role of smoking duration in susceptibility to cutaneous melanoma and MetM. Past-smoking for ≥20 years resulted in fourfold risk factor for melanoma development with respect to healthy controls (OR=4.36) and fivefold risk factor for development of MetM with respect to NMetM (OR=5.31), whereas ≥20 years of smoking ever in life yielded OR=2.43 and OR=2.39, respectively. We also observed that ≥20 years of ever in life smoking combined with certain genetic traits, specifically, with bb, Bb+bb (b allele carriers), Ff, and Ff+ff (f allele carriers) are associated with significant crude ORs ranging from 4 to 9 for MetM cases *vs* healthy controls. Thus, smoke effects in melanoma can be modulated by VDR activity and by the pleiotropic vitamin D endocrine system[11-13,44]. Further studies are necessary to substantiate this significant and complex issue[10,11,44].

Despite the large number of studies[23,24,26-28], results on association of smoking with melanoma still present inconsistencies[23]. Some authors demonstrated risk effects of smoking in melanoma[10,23]. Using multivariate analysis (adjusted for age, sex, site of primary melanoma, and Breslow's thickness), a recent study by Newton-Bishop et al.[10] revealed that smoking duration at diagnosis (hazard ratio=1.11, 95% CI=1.03–1.20, P=0.009) is associated with risk of death from melanoma; and that lower vitamin D levels and smoking are associated with ulceration (a well-known poor prognostic factor) of primary melanomas and poor melanoma-specific survival[10]. We also noted positive association of ≥20 years of smoking with ulceration among melanoma patients. We similarly observed association of ≥20 years of smoking with TIL absence, a finding that predisposes in our and other studies to metastatic melanoma[8,9]. Conversely, other authors showed inverse relationship of smoking with melanoma[26-28]. Multiple potential confounders and biases can explain those protective associations of smoking[23]. Our present findings suggest that effects of smoking duration may be modulated by specific genetic traits.

In our study, past smokers among melanoma cases were twofold more frequent than among healthy controls. Our findings show association of ≥20 years of smoking with increased risk of melanoma, indicating the need for detailed assessment of lifelong smoke duration. We observed that among past smokers, MetM patients smoked approximately 8 years longer than NMetM patients (24.5±13.9 *vs*. 16.4±10.6 years). Notably, we demonstrated that ≥20 years of smoking serves as a two- to fivefold risk factor for MetM compared with NMetM patients. Among study participants, over 80% of past smokers with melanoma quit smoking before cancer diagnosis, with an average of 16 years before melanoma

development. This finding implies that exposure to smoke carcinogens requires long periods to induce melanoma onset and/or metastatic stage. Smoking effects long after smoking discontinuation provide intriguing evidence, which implies that some irreversible damages occur several years before first melanoma diagnosis. Long-lasting variations induced by smoking may include epigenetic changes in specific genes that can remain, for example, differentially methylated after smoking cessation (up to 22 years, as demonstrated by Ambatipudi et al.[47] and/or body accumulation of substances, such as heavy metals, including radionuclides Lead-210 and Polonium-210[48,49]. Explanation for such smoking phenomena require future detailed biological research and human studies. A recent study on melanoma cells observed a role for epigenetic mechanisms in VDR-miRNAs regulation[50].

In our study, ≥20 years of smoking combined with carriage of b allele (Bb+bb) showed adjusted OR=3 for MetM *vs*. NMetM patients after extensive multivariate analyses. This issue warrants further large-scale studies.

Coffee

We did not observe any significant findings in terms of coffee consumption. Thus, our data do not confirm protective effects of coffee consumption, as observed by other researchers[29].

Obesity

In our study, obesity presented an almost fourfold risk factor for melanoma susceptibility (OR=3.54), similar to previous population studies on malignant melanoma[26,33]. Obesity yielded an adjusted OR=5 for all melanoma patients *vs*. healthy controls and adjusted OR=8 for MetM patients *vs*. healthy controls. Obesity combined with Ff or Ff+ff exhibited high adjusted OR=15 and OR=18, respectively, for MetM patients *vs*. healthy controls. BMI is extensively evaluated in relation to several cancer types[51]. A large-cohort Italian study demonstrated that BMI≥25 kg/m^2 is associated with Breslow's thickness>1 mm among melanoma patients[9].

We are the first research group to assess the role of combination of obesity with specific *VDR* genetic traits in cutaneous melanoma. Interpretation of the association of obesity with melanoma may feature a biological rationale. Newton-Bishop et al.[10] hypothesized that inflammation associated with obesity can influence outcome of melanoma. Some evidence also showed the genetic link between obesity and pigmentation or hair color[52].

Triple combination of *VDR* genetic traits, smoking, and obesity

In our study, the highest ORs were observed after combination of a *VDR* genetic trait (b allele carriers) and two lifestyle parameters, i.e., Bb+bb plus ≥20 years of smoking and plus obesity by comparing all melanoma patients *vs*. healthy controls (OR=9.65), MetM patients *vs*. healthy controls (OR=21.6), and MetM *vs*. NMetM patients (OR=12.2). All data remained significant according to multivariate analyses.

However, we failed to calculate ORs for analogous triple combination comprising f allele carriers, because six study subjects with Ff+ff plus ≥20 years of smoking and plus obesity were all MetM patients. Further large-scale studies are necessary for such assessments.

Roles of vitamin D in melanoma require further studies. Melanoma cell culture and xenograft experiments in mice highlighted that vitamin D poses tumoral and metastasis suppression effects[53-55]. Virtually all actions of vitamin D occur through VDR activation. Thus, any modification of VDR activities induced by *VDR* polymorphisms can affect vitamin D functions[13]. Deletion of VDR results in increased susceptibility to tumor formation and reduces ability of keratinocytes to clear UVB-induced DNA mutations[13,56]. VDR can bind to thousands of VDREs on human genome and up- or down-regulate hundreds of genes. Of interest, recent evidence showed a crosstalk between VDR and immune factors[57]. VDR cistrome analyses suggested that altered expression of VDR in colon cancer changes actions of VDR, thus affecting patient outcome[58]. A recent study showed that *VDR* genetic traits can modulate VDR protein expression in excised human melanoma tissues, which might have implications for effects of vitamin D activity on melanoma cells[59].

Thus, future research should focus on complex gene interactions and biological pathways related to vitamin D, VDR, smoking, excessive fat, and environmental factors with melanoma. Improved comprehension of biomolecular pathways will support further progress in melanoma management[60].

Study limitations and strengths

Limitations of our study include limited number of melanomas and high CIs for some categorical variables. Nonetheless, several ORs were statistically significant. Analysis by data stratification for combined variables in some cases resulted in comparison of groups with less than 10

subjects. Thus, future large-scale studies are necessary to better assess the role of such combined variables. We focused on white residents in northern Italy. Thus, our results cannot be generalized to populations with different genetic backgrounds. By contrast, a critical strength of our study is highly defined ethnic background of subjects. This variable bears significance in genetic studies. Variability in racial distribution and genetic melanoma susceptibility among (and across) different countries suggests that melanoma studies should be performed in restricted and well-characterized ethnic groups[7]. Another strength of our study is the detailed reported information, including combinations of genetic and lifestyle factors.

Conclusions

Treatment-resistant metastatic cancer is the most significant contributor to cancer mortality worldwide. Thus, better understanding of factors contributing to development of metastatic cancer may increase likelihood of future improvements in patient management. Our data highlighted that in terms of *VDR* gene alteration by SNPs, vitamin D homeostasis plays roles in cutaneous melanoma and MetM, and these functions are further enhanced by individual smoking habits and BMI. Thus, our findings support a gene-environment contribution to development of malignant melanoma, suggesting the value of genetic screening, smoking cessation[10], and excessive fat prevention[51].

We first suggest gene-environment effects, including smoking duration and obesity, and *VDR* genetic polymorphisms with cutaneous malignant melanoma in general and specifically with MetM. Current data may contribute to development of a personalized/precision management for melanoma patients. Such management may include screening of *VDR* polymorphisms and detailed assessment of smoking habits and BMI. Further investigations are necessary to substantiate and extend our findings to examine different ethnic groups and to identify biological pathways related to vitamin D, smoking, and excessive fat, which influence skin cancers.

Acknowledgements

The authors would like to thank all melanoma patients and healthy controls who participated in the study. The authors are also grateful to Silvio Brusaferro, Dionisio Cauci, Maria Pia Iustulin, and Renato Picco for their help in enrollment of healthy subjects and technicians Patrizia Nacci and Luca Bazzichetto (University of Udine) for their help in experimental analyses. Lastly, the authors would like to express their gratitude to Blase Billack (St. John's University, NY, USA) for critical reading of the manuscript.

Conflict of interest statement

No potential conflicts of interest are disclosed.

References

1. GLOBOCAN 2012. Estimated cancer incidence, mortality and prevalence worldwide in 2012. World Health Organization and International Agency for Research on Cancer (IARC). Available at: http://globocaniarcfr. [Last accessed May 19, 2017].
2. Erdmann F, Lortet-Tieulent J, Schüz J, Zeeb H, Greinert R, Breitbart EW, et al. International trends in the incidence of malignant melanoma 1953-2008-are recent generations at higher or lower risk? Int J Cancer. 2013; 132: 385-400.
3. Arnold M, Holterhues C, Hollestein LM, Coebergh JWW, Nijsten T, Pukkala E, et al. Trends in incidence and predictions of cutaneous melanoma across Europe up to 2015. J Eur Acad Dermatol Venereol. 2014; 28: 1170-8.
4. EUCAN (2912). Malignant melanoma of the skin. Available at: ecoiarcfr/eucan/Canceraspx?Cancer=20.2012: [Last accessed February 3, 2017]
5. AIRTUM. The Italian Association of Cancer Registries. I numeri del cancro in Italia-2016. Available at: http://registri-tumori.it/itacan. accessed May 19, 2017.
6. Registro Tumori Regione Friuli Venezia Giulia (FVG) 2010. Available at: www.cro.sanita.fvg.it [Last accessed May 19, 2017]
7. Cecconi L, Busolin A, Barbone F, Serraino D, Chiarugi A, Biggeri A, et al. Spatial analysis of incidence of cutaneous melanoma in the Friuli Venezia Giulia region in the period 1995-2005. Geospat Health. 2016; 11: 422
8. Bonin S, Albano A, di Meo N, Gatti A, Stinco G, Zanconati F, et al. Cutaneous melanoma frequencies and seasonal trend in 20 years of observation of a population characterised by excessive sun exposure. Radiol Oncol. 2015; 49: 379-85.
9. Gandini S, Montella M, Ayala F, Benedetto L, Rossi CR, Vecchiato A, et al. Sun exposure and melanoma prognostic factors. Oncol Lett. 2016; 11: 2706-14.
10. Newton-Bishop JA, Davies JR, Latheef F, Randerson-Moor J, Chan M, Gascoyne J, et al. 25-Hydroxyvitamin D_2/D_3 levels and factors associated with systemic inflammation and melanoma survival in the Leeds melanoma Cohort. Int J Cancer. 2015; 136: 2890-9.
11. Lee SM, Pike JW. The vitamin D receptor functions as a transcription regulator in the absence of 1, 25-dihydroxyvitamin D_3. J Steroid Biochem Mol Biol. 2016; 164: 265-70.
12. Gandini S, Gnagnarella P, Serrano D, Pasquali E, Raimondi S. Vitamin D receptor polymorphisms and cancer. Adv Exp Med Biol. 2014; 810: 69-105.
13. Bikle DD, Oda Y, Tu CL, Jiang Y. Novel mechanisms for the

vitamin D receptor (VDR) in the skin and in skin cancer. J Steroid Biochem Mol Biol. 2015; 148: 47-51.
14. Orlow I, Reiner AS, Thomas NE, Roy P, Kanetsky PA, Luo L, et al. Vitamin D receptor polymorphisms and survival in patients with cutaneous melanoma: a population-based study. Carcinogenesis. 2016; 37: 30-8.
15. Santonocito C, Capizzi R, Concolino P, Lavieri MM, Paradisi A, Gentileschi S, et al. Association between cutaneous melanoma, Breslow thickness and vitamin D receptor *Bsm*I polymorphism. Br J Dermatol. 2007; 156: 277-82.
16. Gapska P, Scott RJ, Serrano-Fernandez P, Mirecka A, Rassoud I, Górski B, et al. Vitamin D receptor variants and the malignant melanoma risk: a population-based study. Cancer Epidemiol. 2009; 33: 103-7.
17. Randerson-Moor JA, Taylor JC, Elliott F, Chang YM, Beswick S, Kukalizch K, et al. Vitamin D receptor gene polymorphisms, serum 25-hydroxyvitamin D levels, and melanoma: UK case-control comparisons and a meta-analysis of published *VDR* data. Eur J Cancer. 2009; 45: 3271-81.
18. Mandelcorn-Monson R, Marrett L, Kricker A, Armstrong BK, Orlow I, Goumas C, et al. Sun exposure, vitamin D receptor polymorphisms *FokI* and *BsmI* and risk of multiple primary melanoma. Cancer Epidemiol. 2011; 35: e105-10.
19. Orlow I, Roy P, Reiner AS, Yoo S, Patel H, Paine S, et al. Vitamin D receptor polymorphisms in patients with cutaneous melanoma. Int J Cancer. 2012; 130: 405-18.
20. Zhao XZ, Yang BH, Yu GH, Liu SZ, Yuan ZY. Polymorphisms in the vitamin D receptor (VDR) genes and skin cancer risk in European population: a meta-analysis. Arch Dermatol Res. 2014; 306: 545-53.
21. Lee YH, Song GG. Vitamin D receptor FokI, BsmI, TaqI, ApaI, and EcoRV polymorphisms and susceptibility to melanoma: a meta-analysis. J BUON. 2015; 20: 235-43.
22. Hou W, Wan XF, Fan JW. Variants Fok1 and Bsm1 on VDR are associated with the melanoma risk: evidence from the published epidemiological studies. BMC Genet. 2015; 16: 14 doi: 10.1186/s12863-015-0163-6..
23. Thompson CA, Zhang ZF, Arah OA. Competing risk bias to explain the inverse relationship between smoking and malignant melanoma. Eur J Epidemiol. 2013; 28: 557-67.
24. Li Z, Wang Z, Yu Y, Zhang H, Chen L. Smoking is inversely related to cutaneous malignant melanoma: results of a meta-analysis. Br J Dermatol. 2015; 173: 1540-3.
25. IARC. International Agency for Research on Cancer. Available at: http://monographs.iarc.fr/ENG/Classification/. accessed May 19, 2017.
26. Odenbro Å, Gillgren P, Bellocco R, Boffetta P, Håkansson N, Adami J. The risk for cutaneous malignant melanoma, melanoma *in situ* and intraocular malignant melanoma in relation to tobacco use and body mass index. Br J Dermatol. 2007; 156: 99-105.
27. Song FJ, Qureshi AA, Gao X, Li T, Han JL. Smoking and risk of skin cancer: a prospective analysis and a meta-analysis. Int J Epidemiol. 2012; 41: 1694-705.
28. DeLancey JO, Hannan LM, Gapstur SM, Thun MJ. Cigarette smoking and the risk of incident and fatal melanoma in a large prospective cohort study. Cancer Causes Control. 2011; 22: 937-42.
29. Veierod MB, Thelle DS, Laake P. Diet and risk of cutaneous malignant melanoma: a prospective study of 50, 757 Norwegian men and women. Int J Cancer. 1997; 71: 600-4.
30. De Hertog SAE, Wensveen CAH, Bastiaens MT, Kielich CJ, Berkhout MJP, Westendorp RGJ, et al. Relation between smoking and skin cancer. J Clin Oncol. 2001; 19: 231-8.
31. Grange F, Barbe C, Aubin F, Lipsker D, Granel-Brocard F, Velten M, et al. Clinical and sociodemographic characteristics associated with thick melanomas. A population-based, case-case study in France. Arch Dermatol. 2012; 148: 1370-6.
32. Zinkhan M, Stang A, Jöckel KH, Marr A, Bornfeld N, Schmidt-Pokrzywniak A. Having children, social characteristics, smoking and the risk of uveal melanoma: a case-control study. Ophthalmic Epidemiol. 2013; 20: 360-8.
33. Jiang AJ, Rambhatla PV, Eide MJ. Socioeconomic and lifestyle factors and melanoma: a systematic review. Br J Dermatol. 2015; 172: 885-915.
34. Grignol VP, Smith AD, Shlapak D, Zhang X, Del Campo SM, Carson WE. Increased visceral to subcutaneous fat ratio is associated with decreased overall survival in patients with metastatic melanoma receiving anti-angiogenic therapy. Surg Oncol. 2015; 24: 353-8.
35. Renehan AG, Tyson M, Egger M, Heller RF, Zwahlen M. Body-mass index and incidence of cancer: a systematic review and meta-analysis of prospective observational studies. Lancet. 2008; 371: 569-78.
36. Balch CM, Gershenwald JE, Soong SJ, Thompson JF, Atkins MB, Byrd DR, et al. Final version of 2009 AJCC melanoma staging and classification. J Clin Oncol. 2009; 27: 6199-206.
37. Fitzpatrick TB. The validity and practicality of sun-reactive skin types I through VI. Arch Dermatol. 1988; 124: 869-71.
38. Colombini A, Brayda-Bruno M, Ferino L, Lombardi G, Maione V, Banfi G, et al. Gender differences in the *VDR*-FokI polymorphism and conventional non-genetic risk factors in association with lumbar spine pathologies in an Italian case-control study. Int J Mol Sci. 2015; 16: 3722-39.
39. Colombini A, Brayda-Bruno M, Lombardi G, Croiset SJ, Ceriani C, Buligan C, et al. BsmI, ApaI and TaqI polymorphisms in the vitamin D receptor gene (*VDR*) and association with lumbar spine pathologies: An Italian case-control study. PLoS One. 2016; 11: e0155004
40. Colombini A, Brayda-Bruno M, Lombardi G, Croiset SJ, Vrech V, Maione V, et al. FokI polymorphism in the vitamin D receptor gene (*VDR*) and its association with lumbar spine pathologies in the Italian population: a case-control study. PLoS One. 2014; 9: e97027 doi: 10.1371/journal.pone.0097027..
41. Colombini A, Cauci S, Lombardi G, Lanteri P, Croiset S, Brayda-Bruno M, et al. Relationship between vitamin D receptor gene (*VDR*) polymorphisms, vitamin D status, osteoarthritis and intervertebral disc degeneration. J Steroid Biochem Mol Biol. 2013;

138: 24-40.
42. Collins FS, Varmus H. A new initiative on precision medicine. N Engl J Med. 2015; 372: 793-5.
43. Uitterlinden AG, Fang Y, Van Meurs JBJ, Pols HAP, Van Leeuwen JPM. Genetics and biology of vitamin D receptor polymorphisms. Gene. 2004; 338: 143-56.
44. Lee SM, Riley EM, Meyer MB, Benkusky NA, Plum LA, DeLuca HF, et al. 1,25-Dihydroxyvitamin D_3 controls a cohort of vitamin D receptor target genes in the proximal intestine that is enriched for Calcium-regulating components. J Biol Chem. 2015; 290: 18199-215.
45. Zeljic K, Kandolf-Sekulovic L, Supic G, Pejovic J, Novakovic M, Mijuskovic Z, et al. Melanoma risk is associated with vitamin D receptor gene polymorphisms. Melanoma Res. 2014; 24: 273-9.
46. Denzer N, Vogt T, Reichrath J. Vitamin D receptor (VDR) polymorphisms and skin cancer: a systematic review. Dermatoendocrinol. 2011; 3: 205-10.
47. Ambatipudi S, Cuenin C, Hernandez-Vargas H, Ghantous A, Le Calvez-Kelm F, Kaaks R, et al. Tobacco smoking-associated genome-wide DNA methylation changes in the EPIC study. Epigenomics. 2016; 8: 599-618.
48. Taroni M, Zagà V, Bartolomei P, Gattavecchia E, Pacifici R, Zuccaro P, et al. 210Pb and 210Po concentrations in Italian cigarettes and effective dose evaluation. Health Phys. 2014; 107: 195-9.
49. Winters TH, Di Franza JR. Radioactivity in cigarette smoking. N Engl J Med. 1982; 306: 364-5.
50. Essa S, Reichrath S, Mahlknecht U, Montenarh M, Vogt T, Reichrath J. Signature of VDR miRNAs and epigenetic modulation of vitamin D signaling in melanoma cell lines. Anticancer Res. 2012; 32: 383-9.
51. Arnold M, Leitzmann M, Freisling H, Bray F, Romieu I, Renehan A, et al. Obesity and cancer: an update of the global impact. Cancer Epidemiol. 2016; 41: 8-15.
52. Li X, Liang LM, Zhang MF, Song FJ, Nan HM, Wang LE, et al. Obesity-related genetic variants, human pigmentation, and risk of melanoma. Hum Genet. 2013; 132: 793-801.
53. Yudok K, Matsuno H, Kimura T. 1α, 25-dihydroxyvitamin D_3 inhibits in vitro invasiveness through the extracellular matrix and in vivo pulmonary metastasis of B16 mouse melanoma. J Lab Clin Med. 1999; 133: 120-8.
54. Evans SR, Houghton AM, Schumaker L, Brenner RV, Buras RR, Davoodi F, et al. Vitamin D receptor and growth inhibition by 1, 25-dihydroxyvitamin D_3 in human malignant melanoma cell lines. J Surg Res. 1996; 61: 127-33.
55. Eisman JA, Barkla DH, Tutton PJ. Suppression of *in vivo* growth of human cancer solid tumor xenografts by 1, 25-dihydroxyvitamin D_3. Cancer Res. 1987; 47: 21-5.
56. Danielsson C, Törmä H, Vahlquist A, Carlberg C. Positive and negative interaction of 1, 25-dihydroxyvitamin D_3 and the retinoid CD437 in the induction of human melanoma cell apoptosis. Int J Cancer. 1999; 81: 467-70.
57. Singh PK, Van Den Berg PR, Long MD, Vreugdenhil A, Grieshober L, Ochs-Balcom HM, et al. Integration of VDR genome wide binding and GWAS genetic variation data reveals co-occurrence of VDR and NF-κB binding that is linked to immune phenotypes. BMC Genomics. 2017; 18: 132 doi: 10.1186/s12864-017-3481-4..
58. Long MD, Campbell MJ. Integrative genomic approaches to dissect clinically-significant relationships between the VDR cistrome and gene expression in primary colon cancer. J Steroid Biochem Mol Biol. 2016, doi: 10.1016/j.jsbmb.2016.12.013.
59. La Marra F, Stinco G, Buligan C, Chiriacò G, Serraino D, Di Loreto C, et al. Immunohistochemical evaluation of vitamin D receptor (VDR) expression in cutaneous melanoma tissues and four VDR gene polymorphisms. Cancer Biol Med. 2017; 14: 162-75.
60. Rescigno T, Micolucci L, Tecce MF, Capasso A. Bioactive nutrients and nutrigenomics in age-related diseases. Molecules. 2017; 22: 105 doi: 10.3390/molecules22010105..

Cite this article as: Cauci S, Maione V, Buligan C, Linussio M, Serraino D, Stinco G, et al. BsmI (rs1544410) and FokI (rs2228570) vitamin D receptor polymorphisms, smoking, and body mass index as risk factors of cutaneous malignant melanoma in northeast Italy. Cancer Biol Med. 2017; 14: 302-18. doi: 10.20892/j.issn.2095-3941.2017.0064

Cancer Biol Med 2017. doi: 10.20892/j.issn.2095-3941.2017.0020

ORIGINAL ARTICLE

Immunohistochemical evaluation of vitamin D receptor (VDR) expression in cutaneous melanoma tissues and four VDR gene polymorphisms

Francesco La Marra[1], Giuseppe Stinco[1,2], Cinzia Buligan[1,2], Giovanni Chiriacò[1], Diego Serraino[3], Carla Di Loreto[1], Sabina Cauci[1]

[1]Department of Medical Area, School of Medicine, University of Udine, Udine 33100, Italy; [2]Dermatology Clinic, Udine University-Hospital, University of Udine, Udine 33100, Italy; [3]Unit of Cancer Epidemiology, CRO Aviano National Cancer Institute, Aviano 33081, Italy

ABSTRACT

Objective: Vitamin D receptor (VDR) mediates vitamin D activity. We examined whether VDR expression in excised melanoma tissues is associated with VDR gene (*VDR*) polymorphisms.

Methods: We evaluated VDR protein expression (by monoclonal antibody immunostaining), melanoma characteristics, and carriage of *VDR*-FokI-rs2228570 (C>T), *VDR*-BsmI-rs1544410 (G>A), *VDR*-ApaI-rs7975232 (T>G), and *VDR*-TaqI-rs731236 (T>C) polymorphisms (by restriction fragment length polymorphism). Absence or presence of restriction site was denoted by a capital or lower letter, respectively: "F" and "f" for FokI, "B" and "b" for BsmI, "A" and "a" for ApaI, and "T" and "t" for TaqI endonuclease. Seventy-four Italian cutaneous primary melanomas (52.1±12.7 years old) were studied; 51.4% were stage I, 21.6% stage II, 13.5% stage III, and 13.5% stage IV melanomas. VDR expression was categorized as follows: 100% positive *vs*. <100%; over the median 20% (high VDR expression) *vs*. ≤20% (low VDR expression); absence *vs*. presence of VDR-expressing cells.

Results: Stage I melanomas, Breslow thickness of <1.00 mm, level II Clark invasion, Aa heterozygous genotype, and AaTT combined genotype were more frequent in melanomas with high *vs*. low VDR expression. Combined genotypes BbAA, bbAa, AATt, BbAATt, and bbAaTT were more frequent in 100% *vs*. <100% VDR-expressing cells. Combined genotype AATT was more frequent in melanomas lacking VDR expression (odds ratio=14.5; *P*=0.025). VDR expression was not associated with metastasis, ulceration, mitosis >1, regression, tumor-infiltrating lymphocytes, tumoral infiltration of vascular tissues, additional skin and non-skin cancers, and melanoma familiarity.

Conclusions: We highlighted that *VDR* polymorphisms can affect VDR expression in excised melanoma cells. Low VDR expression in AATT carriers is a new finding that merits further study. VDR expression possibly poses implications for vitamin D supplementation against melanoma. VDR expression and *VDR* genotype may become precise medicinal tools for melanoma in the future.

KEYWORDS Vitamin D receptor; VDR protein expression; VDR polymorphism; cutaneous melanoma; metastatic melanoma; skin cancer; predictive biomarkers; FokI polymorphism

Introduction

Cutaneous melanoma incidence continually increases in developed countries, particularly in fair-skinned individuals[1-4]. Recent data[5] indicated that prevalence of melanoma in Northern Italy is two fold higher than in Southern Italy and with Central Italy showing prevalence at intermediate level. High incidence rates were particularly registered in the Northeast Friuli-Venezia Giulia region (19.6/100,000/year in men; 16.4/100,000/year in women)[6], implying that geographically detailed studies should be performed regarding melanoma risk factors[7].

Melanoma is the leading cause of mortality among skin cancer patients with low survival rates[8]. Although new therapeutic treatments are available[9], early detection and surgery remain the main treatment options. Therefore, every new discovery on melanoma biological pathways presents opportunity in improving management and treatment options. Considerable preclinical and epidemiologic data suggest that vitamin D may play an important role in cancer

Correspondence to: Sabina Cauci
E-mail: sabina.cauci@uniud.it
Received February 24, 2017; accepted April 5, 2017.
Available at www.cancerbiomed.org

pathogenesis and progression[10]. Numerous preclinical studies specifically indicated that exposure of cancer cells to high concentrations of vitamin D metabolites halts progression through the cell cycle, induces apoptosis, and slows down or stops tumor growth[11]. Vitamin D also enhances antitumor activity of some cytotoxic anticancer agents in *in vivo* preclinical models[12]. Anti-proliferative effects of vitamin D for cancer prevention and treatment were explored in several malignancies, including skin, breast, prostate, colorectal, and other cancers[8,13-18]. Numerous epidemiological studies supported the hypothesis that individuals with lower serum vitamin D levels feature higher exposure risk to different cancers[12]. Current literature suggests the chemopreventive role of vitamin D by acting against initiation and progression of tumorigenesis[8,12-18]. Despite the stronger consensus on protective role of vitamin D against cancer, particularly by reduction of mortality rate, its therapeutic function for cancer patients remains debatable[10,19]. Remarkably, a recent meta-analysis showed that vitamin D supplementation minimally affects total cancer incidence, even when total cancer mortality is significantly reduced[20].

Vitamin D receptor (VDR) is a nuclear transcription factor belonging to the nuclear receptor superfamily that binds 1α, 25-dihydroxyvitamin D (calcitriol) with high affinity and specificity[19]. Upon binding to the active form of vitamin D, VDR translocates from cytoplasm into the nucleus and binds to vitamin D responsive elements (VDREs), thus up- or down-regulating hundreds of genes directly controlled by vitamin D[19,21,22]. Increasing evidence showed pleiotropic hormonal effects of vitamin D on calcium and skeletal metabolism, immunological responses, detoxification, oxidative stress, cancer-related metabolic pathways, proliferation, and cell differentiation[18,19,21].

VDR is abundantly expressed in the skin[19,21]. Some VDR expression was also reported to occur in cultured melanoma cells[11,13,14,23]. Intermittent sun exposure or ultraviolet (UV) radiation and sunburns are known environmental risk factors for melanoma[24,25]. However, chronic and continuous UV radiation exposure activates vitamin D biosynthesis, which in turn can develop a protective action against tumoral proliferation[8,12,16,25]. Four recently discovered mechanisms may underlie actions of VDR as a tumor suppressor in the skin[10].

Human *VDR* gene is located on chromosome 12q12-q14 and comprises 11 exons and 11 introns[26]. Genetic variants of *VDR* may modulate its actions, with FokI, BsmI, ApaI, and TaqI being the most studied single-nucleotide polymorphisms (SNPs)[26-28]. *VDR*-FokI polymorphism is a functional SNP that extends lengths of the receptor protein from 424 to 427 amino acid residues. BsmI, ApaI, and TaqI polymorphisms are located in the 3′ terminal region of the *VDR* gene and do not affect protein sequence of the VDR receptor. FokI polymorphism is reported not to be in linkage disequilibrium with the other three polymorphisms. Instead, BsmI, ApaI, and TaqI polymorphisms are reported to be in linkage disequilibrium to a variable extent; thus, combined genotypes including two or three of these polymorphisms were investigated in literature[26,28,29]. Some evidence suggested that genotypes FF, BB, tt, and the combined genotype BBAAtt may be associated with increased expression of VDR, which in turn regulates actions of vitamin D[26-28]. Roles of *VDR* polymorphisms in melanoma were evaluated in some recent studies and meta-analyses[30-37]. However, associations of *VDR* polymorphisms with skin cancer risk remain insufficiently characterized[30,31,36].

At present, no study examined *VDR* polymorphisms and VDR expression in melanoma cells of excised tissues from patients.

Thus far, only one cohort of 69 Polish melanoma patients was investigated by two studies for VDR expression in tumor tissues[38,39]. VDR expression progressively decreases from normal skin to melanocytic nevi to melanomas[38], suggesting the relationship between VDR expression and melanoma prognosis[39]. Brożyna and colleagues[38] observed reduced expression levels of VDR in skin surrounding nevi and melanomas as opposed to normal skin.

Advances in melanoma treatment can be achieved through developments in understanding of melanoma risk factors, genomics, and molecular pathogenesis[31,40].

By immunohistochemical staining of primary cutaneous melanoma tissues, we investigated VDR expression in relation to characteristics, melanoma histological grading, and metastatic stage of patients. We also explored the association of four *VDR* SNPs- FokI-rs2228570 C>T located in exon 2, BsmI-rs1544410 G>A located in intron 8, ApaI-rs7975232 T>G located in intron 8, and TaqI-rs731236 T>C located in exon 9- with VDR expression levels in cutaneous malignant melanoma tissues.

Patients and methods

Patients

Enrolment and clinical visits of all study participants were performed at the Udine University-Hospital Dermatology Clinic. Diagnostic procedures were conducted according to routine protocols. All participants signed a written informed

consent. The Udine Institutional Ethical Committee approved the study protocol in accordance to the Declaration of Helsinki.

Seventy-four (39 males, 35 females, age range: 29-82 years) unrelated patients (hospitalized or outpatients) who consecutively underwent surgical excision of cutaneous melanoma were enrolled based on a retrospective design. Inclusion criteria were as follows: melanoma different from that in *in situ* only, absence of mucosal melanomas, patient is a resident of Friuli-Venezia Giulia region (Northern Italy), and absence of major chronic diseases, such as autoimmune diseases, and type 1 diabetes.

Assessment of melanoma diagnosis and patient stage classification were performed by clinical/histological findings as described by Balch et al.[41]. For patients with multiple melanomas, we examined only the first main melanoma according to histological assessment of major primary tumor grading, and primary melanoma characteristics were accounted for study analyses.

Questionnaires were used to collect information from each participant; data obtained included demographic and lifestyle characteristics and medical and family history of melanoma. Body mass index (BMI) was determined by ratio of weight (kg) to squared height (m).

Immunohistochemical staining and evaluation of VDR expression

Slides stained with hematoxylin and eosin were reviewed for each case from formalin-fixed and paraffin-embedded blocks and were selected for VDR immunohistochemical staining. Immunohistochemistry was performed on 5 μm thick paraffin sections as follows: after dewaxing, rehydration and endogenous peroxidase quenching with 3% v/v H_2O_2 in methanol for 15 min, antigen retrieval in 0.01 M citrate buffer at 98°C water bath for 40 min, application and incubation of primary antibody (VDR mouse monoclonal D-6, sc-13133, Santa Cruz Biotechnology, Texas, USA) at 1:200 dilution 1 h at room temperature[42,43], incubation with peroxidase-based EnVision+ /Horseradish peroxidase (Dako A/S, Glostrup, Denmark) for 30 min at room temperature, and treatment with diaminobenzidine for 3 min[44]. The sections were then counterstained with Mayer's hematoxylin, dehydrated, and mounted. VDR expression was evaluated on tumor cells (nuclear and cytoplasmic staining) of the whole section. Immunolabeled sections were viewed under Nikon Eclipse 80i light microscope at 25× magnification, and a semi-quantitative evaluation was performed to determine VDR expression levels in malignant melanoma cells (nuclear and cytoplasmic staining). Staining of sweat gland cells, which consistently showed strong and diffused positivity throughout all samples, was used as positive reference. The term "emboli" indicates tumoral invasion of vascular cells observed in slide specimens. Melanoma specimens were reviewed by two pathologists involved in the study but were unaware of all other clinical and molecular data during evaluation. Our categorization choices were based on the following considerations: A) when melanomas feature 100% VDR-positive cells, all tumor cells are possibly responsive to vitamin D stimulation; B) when 0% cells are positive for VDR expression, virtually all tumor cells do not respond to vitamin D stimulation; C) aside from extreme conditions, a cut off at median percentage of positive cells can be reasonably used for evaluating the half of samples with higher *vs*. the half of samples with lower VDR expression. Therefore, results were ranked based on percentage of cells positive for VDR expression (irrespective of staining intensity). Cytoplasmic VDR expression ranged from 0% to 100%, with a median value at 20.0%. We categorized variables as follows: 100% cytoplasmic VDR-expression-positive cells versus all remaining melanomas; >20% cytoplasmic VDR-expression-positive cells (high VDR expression) *vs*. ≤20% positive cells (low VDR expression); and absent (0% positive cells) *vs*. present (>0% positive cells) VDR expression. Only 11 out of 74 cases (14.9%) showed nuclear VDR-positive immunostaining, and all these cases were categorized into >20% cytoplasmic VDR-expression-positive cells. Given the low number of melanoma specimens showing VDR expression detected in nuclei, this parameter was not further analyzed.

Determination of *VDR* gene polymorphisms

Determination of SNP *VDR*-FokI (C>T), *VDR*-BsmI (G>A), *VDR*-ApaI (T>G), *VDR*-TaqI (T>C) was performed as previously described[29,45] after extraction of genomic DNA from ethylenediaminetetraacetic-acid -venous blood samples[46]. Absence or presence of restriction site was denoted by a capital or lower letter, respectively: "F" and "f" for FokI, "B" and "b" for BsmI, "A" and "a" for ApaI, and "T" and "t" for TaqI endonucleases[28,29]. FokI, BsmI, ApaI and TaqI polymorphisms of *VDR* were studied using previously tested primers[29,45] to amplify appropriate DNA fragments. FokI enzyme (Euroclone, Milano, Italy) digestion of 265 bp amplified DNA was used to determine FokI restriction fragment length polymorphism (RFLP) yielding 196 and 69 bp fragments in the presence of f allele[46]. To analyze BsmI polymorphism, the resulting amplified 825 bp polymerase

chain reaction (PCR) fragment was digested with BsmI restriction enzyme (Euroclone, Milano, Italy), generating 650 and 175 bp fragments in the presence of b allele[29]. ApaI digestion of the 740 bp amplified DNA was used to determine both ApaI and TaqI RFLP, generating 530 and 210 bp fragments in the presence of a allele. Digestion with TaqI of the 740 bp PCR fragment generated 290, 245, and 205 bp fragments in the presence of t allele and 495 and 245 bp fragments in its absence (T allele) owing to an additional monomorphic TaqI site[29]. DNA fragments were separated by polyacrylamide gel electrophoresis.

Statistical analysis

Kolmogorov-Smirnov test was used to assess normal data distribution. Percentage of VDR-positive cells by immunohistochemical staining was not normally distributed. Thus, median values and ranges were reported for this variable. Mann-Whitney U test was used to assess differences between groups. Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated to determine association of groups with different VDR expression and melanoma characteristics, alleles, genotypes, and combined genotypes. Our study was explorative as no previous investigation determined frequencies of VDR polymorphisms in melanoma patients according to immunohistochemical findings of VDR expression Prior to study enrolment, we evaluated that a number of 70 subjects fitted the 80% power at an alpha level of 0.05 to detect differences between high (above the median) and low VDR expression melanoma groups whether OR value equals 3 or more for a SNP site[47]. Deviation tests from Hardy-Weinberg equilibrium (HWE) were performed using a separate Chi-square distribution for each SNP[29]. Linkage disequilibrium (LD) between SNPs was determined as described[29]. Two-sided significance level was set at 0.05, and P values $\leq$ 0.10 were considered as a tendency to be significant. Statistical software SPSS (SPSS Inc., Chicago, IL, USA) was used for statistical analyses.

Results

VDR immunohistochemical staining

A significant variability in VDR immunohistochemical staining was observed in cytoplasm of melanoma cells, with 16.2% (12/74) of patients tested positive for 100% melanoma cell staining, whereas 20.3% (15/74) were tested negative. Median percentage value of VDR-positive staining reached 20.0%. **Figures 1**, **2**, and **3** display representative images of VDR immunohistochemical staining in excised tissues from patients with primary cutaneous melanoma, showing 100% staining, intermediate staining, and no staining, respectively.

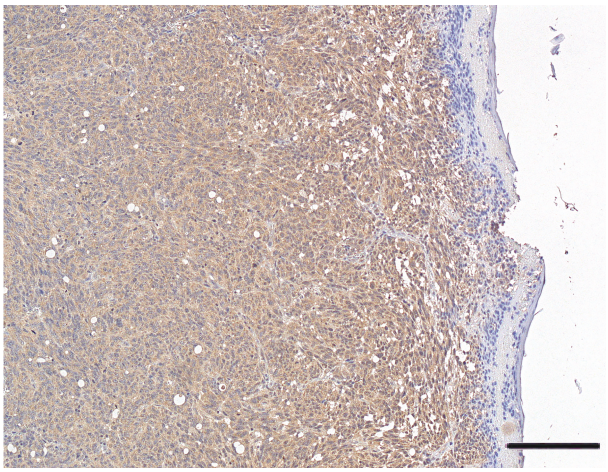


Figure 1 Representative image of VDR protein expression in cutaneous melanoma tissue. Melanoma showing diffuse positivity for VDR expression. Eroded epidermis (H&E staining, 25×. Bar indicates 50 μm).

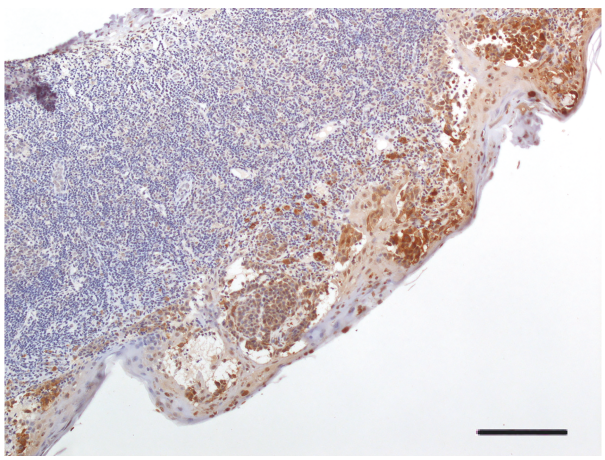


Figure 2 Representative image of VDR protein expression in cutaneous melanoma tissue. Melanoma featuring tumor-infiltrating lymphocytes (TILs). Intraepidermal component of lesion (at the bottom of image) shows strong positivity for VDR expression (H&E staining, 25×. Bar indicates 50 μm).

Patients and melanoma characteristics according to VDR expression

All 74 (35 females and 39 males) cutaneous melanoma patients were white residents in Northern Italy. Average age at melanoma diagnosis was 52.1±12.7 years, with 27%

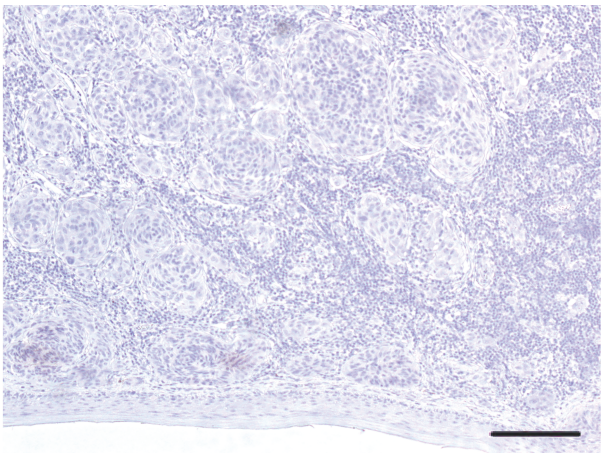


Figure 3 Representative image of VDR protein expression in cutaneous melanoma tissue. Melanoma featuring TILs. In this case, melanoma cells nesting in the dermis are negative throughout the whole lesion (H&E staining, 25×. Bar indicates 50 μm).

(20/74) of patients exhibiting metastatic melanoma (i.e., stages III and IV), and all showed Clark level higher than Clark I invasion.

Table 1 shows the main clinical characteristics of melanoma patients and compares three different binary categories according to percentage of tumor cells positive for VDR staining: a) 100% positive *vs.* <100% positive, b) over 20% (median value) positive (high VDR expression) *vs.* ≤ 20% positive (low VDR expression), and c) absence *vs.* presence of VDR-positive cells. Complete data on ORs and CIs are reported on **Table S1**. Age of melanoma diagnosis, gender, BMI, and smoking did not differ between compared groups. Differences were not significant in comparison of 12 subjects with 100% VDR-positive cells *vs.* 62 subjects with <100% VDR-positive cells. The following statically significant differences were observed during comparison of 36 melanomas with high VDR expression *vs.* 38 melanomas with low VDR expression: stage I was more frequent in the former (63.9% *vs.* 39.5%, OR=2.71, CI=1.06–6.95, *P*=0.036), whereas stage II was less frequent (11.1% *vs.* 31.6%, OR=0.27, CI=0.08–0.94, *P*=0.033); the former presents a lower mean Breslow thickness (1.23±0.88 *vs.* 2.27±1.97 mm, *P*=0.008) and more frequent Breslow thickness of <1.00 mm (50.0% *vs.* 26.3%, OR=2.80, CI=1.06–7.41, *P*=0.036) and less frequent Breslow thickness ≥1.01 mm (50.0% *vs.* 77.8%, OR=0.36, CI=0.13–0.95, *P*=0.036). A higher frequency of Clark II invasion (38.9% *vs.* 15.8%, OR=3.39, CI=1.13–10.2, *P*=0.025) was observed in high- than low-VDR-expression group. Superficial spreading was present in 61.1% of melanomas with high VDR expression *vs.* 39.5% of low-VDR-expression melanomas, where this difference did not reach significant *P* values (*P*=0.063). In comparing absence *vs.* presence of VDR-positive immunohistochemical staining of melanoma cells, the only significant finding was a higher frequency of stage II A in the former *vs.* the latter (26.7% *vs.* 5.1%, OR=6.79, CI=1.33–34.7, *P*=0.028) (all sub-stage data are shown in **Table S1**).

Overall, as shown in **Table 1**, none of the tumor markers commonly associated with severe prognosis and metastatic stage were associated with VDR immunohistochemical staining; these markers included ulceration, mitosis >1, absence of tumor-infiltrating lymphocytes (TILs), emboli, and epithelioid variants. The presence of multiple melanomas, additional skin, non-skin cancers, and melanoma familiarity did not correlate with VDR immunohistochemical staining.

By further analysis, median VDR expression did not differ between 20 metastatic melanomas (median: 17.5%, range: 0%–100% VDR-positive cells) and 54 non-metastatic melanomas (median: 25.0%, range: 0%–100% VDR-positive cells), with *P*=0.796. Significant *P* value (*P*=0.095) was not observed in differences in median values of VDR expression of stage I melanomas compared with those of stages II+III+IV. Median values of VDR expression of Clark II (in our cohort, none of melanomas showed Clark I invasion) melanomas were significantly higher compared with Clark III+IV+V levels (median: 70.0%, range: 0%–100% *vs.* median: 10.0%, range: 0%–100% VDR-positive cells, *P*=0.019). A significant *P* value was not observed in differences in median values of VDR expression in superficially spreading melanomas compared with the remaining ones (*P*=0.075).

Patient and melanoma characteristics according to *VDR* polymorphisms

Table 2 shows *VDR* polymorphism genotypes, alleles, and combined genotype frequencies in all 74 patients and in groups of melanomas categorized according to VDR immunohistochemical staining. Each *VDR* polymorphism of FokI, BsmI, ApaI, and TaqI was in HWE. FokI SNP was not in LD with other SNPs. BsmI was in LD with ApaI and TaqI, and ApaI was in LD with TaqI. Thus, for further analyses, we considered binary and ternary combination of genotypes comprising BsmI, ApaI and TaqI polymorphisms. As observed in other studies[26,29], not all theoretically possible binary and ternary combination of genotypes were observed; thus, **Table 2** reports only combined genotypes with at least one confirmed finding.

Differences between VDR expression groups were not

Table 1 Clinical characteristics of 74 consecutively enrolled melanoma patients and comparison between groups of 100% VDR-positive cells (*n*=12) *vs*. <100% (*n*=62); over the median (>20%) VDR-positive cells (*n*=36) *vs*. below or equal the median (≤20%) (*n*=38); and absence of VDR-positive cell (*n*=15) *vs*. remaining cases with detected VDR expression (*n*=59)

Characteristics	All melanoma patients (*n*=74)	100% VDR-positive cells (*n*=12)	<100% VDR-positive cells (*n*=62)	*P*=100% *vs*. <100% VDR-positive	>20%[a] VDR-positive cells (*n*=36)	≤20% VDR-positive cells (*n*=38)	*P*>20% *vs*. ≤ 20% VDR-positive	VDR absence (0% positive) (*n*=15)	VDR presence (>0% positive) (*n*=59)	PVDR absence *vs*. presence
Age at melanoma diagnosis, years, mean ± SD	52.1±12.7	49.8±13.0	52.7±12.9	0.519[a]	51.7±13.9	52.8±12.0	0.770[a]	51.5±14.5	52.5±12.6	0.767[a]
Age <50 years at melanoma diagnosis	31 (41.9)	6 (50.0)	25 (40.3)	0.534	16 (44.4)	15 (39.5)	0.665	7 (46.7)	24 (40.7)	0.675
BMI, kg/m^2, mean ± SD	25.8±4.0	25.0±4.6	25.9±3.9	0.412[a]	26.0±4.3	25.6±3.8	0.804[a]	25.7±2.6	25.8±4.3	0.762[a]
Male	39 (52.7)	6 (50.0)	33 (53.2)	0.838	20 (55.6)	19 (50.0)	0.632	9 (60.0)	30 (50.8)	0.526
Smoker	11 (14.9)	2 (16.7)	9 (14.5)	1.000	4 (11.1)	7 (18.4)	0.377	2 (13.3)	9 (15.3)	1.000
Stage I	38 (51.4)	6 (50.0)	32 (51.6)	0.919	23 (63.9)	15 (39.5)	0.036	6 (40.0)	32 (54.2)	0.325
Stage II	16 (21.6)	2 (16.7)	14 (22.6)	1.000	4 (11.1)	12 (31.6)	0.033	6 (40.0)	10 (16.9)	0.077
Stage III	10 (13.5)	3 (25.0)	7 (11.3)	0.351	6 (16.7)	4 (10.5)	0.510	1 (6.7)	9 (15.3)	0.676
Stage IV	10 (13.5)	1 (8.3)	9 (14.5)	1.000	3 (8.3)	7 (18.4)	0.310	2 (13.3)	8 (13.6)	1.000
Metastatic melanoma (Stage III +IV)	20 (27.0)	4 (33.3)	16 (25.8)	0.724	9 (25.0)	11 (28.9)	0.702	3 (20.0)	17 (28.8)	0.746
Trunk	47 (63.5)	5 (41.7)	42 (67.7)	0.108	24 (66.7)	23 (60.5)	0.583	7 (46.7)	40 (67.8)	0.129
Upper limb	6 (8.1)	2 (16.7)	4 (6.5)	0.249	2 (5.6)	4 (10.5)	0.675	3 (20.0)	3 (5.1)	0.093
>Lower limb	14 (18.9)	3 (25.0)	11 (17.7)	0.687	7 (19.4)	7 (18.4)	0.911	4 (26.7)	10 (16.9)	0.463
Hands/feet	5 (6.8)	2 (16.7)	3 (4.8)	0.183	2 (5.6)	3 (7.9)	1.000	0 (–)	5 (8.5)	0.576
Head/neck	2 (2.7)	0 (–)	2 (3.2)	1.000	1 (2.8)	1 (2.6)	1.000	1 (6.7)	1 (1.7)	0.367
Superficial spreading	37 (50.0)	7 (58.3)	30 (48.4)	0.528	22 (61.1)	15 (39.5)	0.063	6 (40.0)	31 (52.5)	0.386
Nodular	31 (41.9)	4 (33.3)	27 (43.5)	0.512	12 (33.3)	19 (50.0)	0.146	8 (53.3)	23 (39.0)	0.314
Acral lentiginous	3 (4.1)	1 (8.3)	2 (3.2)	0.417	1 (2.8)	2 (5.3)	1.000	0 (–)	3 (5.1)	1.000
Spitzoide	2 (2.7)	0 (–)	2 (3.2)	1.000	1 (2.8)	1 (2.6)	1.000	1 (6.7)	1 (1.7)	0.367
Others	3 (4.1)	0 (–)	3 (4.8)	1.000	0 (–)	3 (7.9)	0.240	0 (–)	3 (5.1)	1.000
Breslow thickness, mm, mean ± SD	1.77±1.62	1.34±0.94	1.85±1.71	0.416[a]	1.23±0.88	2.27±1.97	0.008[a]	2.16±1.88	1.66±1.55	0.145[a]

Continued

Continued

Characteristics	All melanoma patients (*n*=74)	100% VDR-positive cells (*n*=12)	<100% VDR-positive cells (*n*=62)	*P*=100% *vs.* <100% VDR-positive	>20%[a] VDR-positive cells (*n*=36)	≤20% VDR-positive cells (*n*=38)	*P*>20% *vs.* ≤ 20% VDR-positive	VDR absence (0% positive) (*n*=15)	VDR presence (>0% positive) (*n*=59)	PVDR absence *vs.* presence
Breslow thickness <1.00 mm	28 (37.8)	4 (33.3)	24 (38.7)	1.000	18 (50.0)	10 (26.3)	0.036	4 (26.7)	24 (40.7)	0.318
Breslow thickness ≥1.01 mm	46 (62.2)	8 (66.7)	38 (61.3)	1.000	18 (50.0)	28 (77.8)	0.036	11 (73.3)	35 (59.3)	0.318
Clark I	0 (–)	0 (–)	0 (–)		0 (–)	0 (–)	–[b]	0 (–)	0 (–)	–[b]
Clark II	20 (27.0)	5 (41.7)	15 (24.2)	0.287	14 (38.9)	6 (15.8)	0.025	2 (13.3)	18 (30.5)	0.328
Clark III	15 (20.3)	1 (8.3)	14 (22.6)	0.439	6 (16.7)	9 (23.7)	0.453	4 (26.7)	11 (18.6)	0.488
Clark IV	36 (48.6)	6 (50.0)	30 (48.4)	0.919	15 (41.7)	21 (55.3)	0.242	8 (53.3)	28 (47.5)	0.684
Clark V	2 (2.7)	0 (–)	2 (3.2)	1.000	0 (–)	2 (5.3)	0.494	1 (6.7)	1 (1.7)	0.367
Ulceration	25 (33.8)	5 (41.7)	20 (32.3)	0.525	11 (30.6)	14 (36.8)	0.568	5 (33.3)	20 (33.9)	0.967
Mitosis >1	51 (68.9)	8 (66.7)	43 (69.4)	1.000	23 (63.9)	28 (73.7)	0.363	12 (80.0)	39 (66.1)	0.365
Regression	12 (16.2)	1 (8.3)	11 (17.7)	0.677	6 (16.7)	6 (15.8)	0.919	4 (26.7)	8 (13.6)	0.248
Brisk positive TILs[c]	25 (33.8)	5 (41.7)	20 (32.3)	0.525	15 (41.7)	10 (26.3)	0.163	5 (33.3)	20 (33.9)	0.967
Non-brisk, TILs[c]	26 (35.1)	3 (25.0)	23 (37.1)	0.522	10 (27.8)	16 (42.1)	0.197	7 (46.7)	19 (32.2)	0.295
TIL absence[c]	23 (31.1)	4 (33.3)	19 (30.6)	1.000	11 (30.6)	12 (31.6)	0.924	3 (20.0)	20 (33.9)	0.365
Emboli	9 (12.2)	2 (16.7)	7 (11.3)	0.633	4 (11.1)	5 (13.2)	1.000	2 (13.3)	7 (11.9)	1.000
Microsatellitosis	2 (2.7)	0 (–)	2 (3.2)		1 (2.8)	1 (2.6)	1.000	0 (–)	2 (3.4)	1.000
Epithelioid variant	11 (14.9)	3 (25.0)	8 (12.9)	0.371	6 (16.7)	5 (13.2)	0.672	0 (–)	11 (18.6)	0.106
Fusate variant	4 (5.4)	0 (–)	4 (6.5)	1.000	3 (8.3)	1 (2.6)	0.351	0 (–)	4 (6.8)	0.576
Small-cell variant	2 (2.7)	0 (–)	2 (3.2)	1.000	1 (2.8)	1 (2.6)	1.000	1 (6.7)	1 (1.7)	0.367
>1 melanoma	9 (12.2)	1 (8.3)	8 (12.9)	1.000	4 (11.1)	5 (13.2)	1.000	2 (13.3)	7 (11.9)	1.000
Additional non-melanoma skin cancer	11 (14.9)	2 (16.7)	9 (14.5)	1.000	6 (16.7)	5 (13.2)	0.672	3 (20.0)	8 (13.6)	0.684
Additional non-skin cancer	14 (18.9)	3 (25.0)	11 (17.7)	0.687	9 (25.0)	5 (13.2)	0.194	3 (20.0)	11 (18.6)	1.000
Melanoma familiarity	13 (17.6)	3 (25.0)	10 (16.1)	0.432	8 (22.2)	5 (13.2)	0.306	1 (6.7)	12 (20.3)	0.282

[a] Two-tailed Mann–Whitney U test. [b] OR uncountable because one or two of compared groups included zero subject. [c] TILs, tumor infiltrating lymphocytes.

Table 2 Genotype and allele of ApaI, VDR-polymorphism, and BsmI-ApaI, ApaI-TaqI, BsmI-ApaI-TaqI combined genotypes of 74 melanoma patients and comparisons between groups of 100% VDR-positive cells (n=12) *vs*. <100% (n=62); over the median (>20%) VDR-positive cells (n=36) *vs*. below or equal the median (≤20%) (n=38); and absence of VDR-positive cell (n=15) *vs*. remaining cases with detected VDR expression (n=59)

VDR genotype or combined genotype	All melanoma patients (n=74)	100% VDR-positive cells (n=12)	<100% VDR-positive cells (n=62)	P=100% *vs*.<100% VDR-positive	>20%[a] VDR-positive cells (n=36)	≤20% VDR-positive cells (n=38)	P>20% *vs*. ≤20% VDR-positive	VDR absence (0% positive) (n=15)	VDR presence (>0% positive) (n=59)	PVDR absence *vs*. presence
ApaI genotype										
AA	30 (40.5)	8 (66.7)	22 (35.5)	0.058	13 (36.1)	17 (44.7)	0.450	8 (53.3)	22 (37.3)	0.258
Aa	36 (48.6)	4 (33.3)	32 (51.6)	0.246	22 (61.1)	14 (36.8)	0.037	5 (33.3)	31 (52.5)	0.184
aa	8 (10.8)	0 (–)	8 (12.9)	0.339	1 (2.8)	7 (18.4)	0.056	2 (13.3)	6 (10.2)	0.660
A allele	96/148 (64.9)	20/24 (83.3)	76/124 (61.3)	0.038	48/72 (66.7)	48/76 (63.2)	0.655	21/30 (70.0)	75/118 (63.6)	0.509
a allele	52/148 (35.1)	4/24 (16.7)	48/124 (38.7)	0.038	24/72 (33.3)	28/76 (36.8)	0.655	9/30 (30.0)	43/118 (36.4)	0.509
BsmI-ApaI combined genotype										
BBAA	23 (31.1)	5 (41.7)	18 (29.0)	0.498	9 (25.0)	14 (36.8)	0.271	6 (40.0)	17 (28.8)	0.533
BbAA	5 (6.8)	3 (25.0)	2 (3.2)	0.028	4 (11.1)	1 (2.6)	0.194	1 (6.7)	4 (6.8)	1.000
BbAa	31 (41.9)	1 (8.3)	30 (48.4)	0.010	18 (50.0)	13 (34.2)	0.169	5 (33.3)	26 (44.1)	0.452
Bbaa	2 (2.7)	0 (–)	2 (3.2)	1.000	0 (–)	2 (5.3)	0.494	0 (–)	2 (3.4)	1.000
bbAA	2 (2.7)	0 (–)	2 (3.2)	1.000	0 (–)	2 (5.3)	0.494	1 (6.7)	1 (1.7)	0.367
bbAa	5 (6.8)	3 (25.0)	2 (3.2)	0.028	4 (11.1)	1 (2.6)	0.194	0 (–)	5 (8.5)	0.576
bbaa	6 (8.1)	0 (–)	6 (9.7)	0.581	1 (2.8)	5 (13.2)	0.200	2 (13.3)	4 (6.8)	0.595
ApaI-TaqI combined genotype										
AATT	4 (5.4)	0 (–)	4 (6.5)	1.000	0 (–)	4 (10.5)	0.115	3 (20.0)	1 (1.7)	0.025
AATt	14 (18.9)	6 (50.0)	8 (12.9)	0.008	9 (25.0)	5 (13.2)	0.194	4 (26.7)	10 (16.9)	0.463
AAtt	12 (16.2)	2 (16.7)	10 (16.1)	1.000	4 (11.1)	8 (21.1)	0.246	1 (6.7)	11 (18.6)	0.439
AaTT	12 (16.2)	3 (25.0)	9 (14.5)	0.399	9 (25.0)	3 (7.9)	0.046	1 (6.7)	11 (18.6)	0.439
AaTt	24 (32.4)	1 (8.3)	23 (37.1)	0.089	13 (36.1)	11 (28.9)	0.511	4 (26.7)	20 (33.9)	0.761
aaTT	8 (10.8)	0 (–)	8 (12.9)	0.339	1 (2.8)	7 (18.4)	0.056	2 (13.3)	6 (10.2)	0.660
BsmI-ApaI-TaqI combined genotype										
BBAATT	1 (1.4)	0 (–)	1 (1.6)	1.000	0 (–)	1 (2.6)	1.000	1 (6.7)	0 (–)	0.203
BBAATt	10 (13.5)	3 (25.0)	7 (11.3)	0.351	5 (13.9)	5 (13.2)	1.000	4 (26.7)	6 (10.2)	0.110
BBAAtt	12 (16.2)	2 (16.7)	10 (16.1)	1.000	4 (11.1)	8 (21.1)	0.246	1 (6.7)	11 (18.6)	0.439
BbAATt	4 (5.4)	3 (25.0)	1 (1.6)	0.012	4 (11.1)	0 (–)	0.051	0 (–)	4 (6.8)	0.576
BbAATT	1 (1.4)	0 (–)	1 (1.6)	1.000	0 (–)	1 (2.6)	1.000	1 (6.7)	0 (–)	0.203
BbAaTT	7 (9.5)	0 (–)	7 (11.3)	0.590	5 (13.9)	2 (5.3)	0.255	1 (6.7)	6 (10.2)	1.000
BbAaTt	24 (32.4)	1 (8.3)	23 (37.1)	0.089	13 (36.1)	11 (28.9)	0.511	4 (26.7)	20 (33.9)	0.761
BbaaTT	2 (2.7)	0 (–)	2 (3.2)	1.000	0 (–)	2 (5.3)	0.494	0 (–)	2 (3.4)	1.000
bbAATT	2 (2.7)	0 (–)	2 (3.2)	1.000	0 (–)	2 (5.3)	0.494	1 (6.7)	1 (1.7)	0.367
bbAaTT	5 (6.8)	3 (25.0)	2 (3.2)	0.028	4 (11.1)	1 (2.6)	0.194	0 (–)	5 (8.5)	0.576
bbaaTT	6 (8.1)	0 (–)	6 (9.7)	0.581	1 (2.8)	5 (13.2)	0.200	2 (13.3)	4 (6.8)	0.595

[a] Over the median value of percentage (%) of cells positive for VDR protein.

significant for single FokI, BsmI, and TaqI genotypes and alleles, and BsmI-TaqI combined genotypes (data shown in **Table S3**).

As reported in **Table 2** (data comprising all ORs and CIs are shown in **Table S2**. **Table S1** to **Table S3** in the supplementary materials, available with the full text of this article at www.cancerbiomed.org), heterozygous Aa genotype was more frequent in melanomas with high than low VDR expression (61.1% *vs*. 36.8%, OR=2.69, CI=1.05–6.90, *P*=0.037). A allele was found in 83.3% of 100% VDR-positive melanomas and in 61.3% of those with <100% VDR-positive cells (OR=3.16, CI=1.02–9.80, *P*=0.038).

By analyzing combined genotypes (**Table 2**), six significant differences were observed after comparing 100% *vs*. <100% VDR-expression-positive groups. Combined genotypes BbAA (OR=10.0, CI=1.46–68.3, *P*=0.028), bbAa (OR=10.0, CI=1.46–68.3, *P*=0.028), AATt (OR=6.75, CI=1.74–26.1, *P*=0.008), BbAATt (OR=20.3, CI=1.90–217, *P*=0.012), and bbAaTT (OR=10.0, CI=1.46–68.3, *P*=0.028) were more frequent in the former *vs*. the latter group, whereas combined genotype BbAa (OR=0.10, CI=0.01–0.80, *P*=0.010) was less frequent in the former group than the latter.

In comparing the >20% *vs*. ≤20% VDR-expression-positive groups, combined genotype AaTT (OR=3.89, CI=0.96–15.8, *P*=0.046) was more frequent in the former than in the latter group.

In comparing absent- *vs*. present-VDR-expression groups, the ApaI-TaqI combined genotype AATT (OR=14.5, CI=1.39–152, *P*=0.025) was more frequent in the melanoma group lacking VDR expression *vs*. the remaining patients.

Considering continuous median percentage values of VDR-expression-positive cells, with 100% VDR-positive cells, significantly higher VDR expression was observed for bbAa (median: 100.0%, range: 15%–100% *vs*. median: 20.0%, range: 0%–100%, *P*=0.029), BbAATt (median: 100.0%, range: 60%–100% *vs*. median: 20.0%, range: 0%–100%, *P*=0.011), bbAaTT (median: 100.0%, range: 15%–100% *vs*. median: 20.0%, range: 0%–100%, *P*=0.029) combined genotype carriers *vs*. non-carriers. By contrast, significantly lower VDR expression was noted for carriers of AATT combined genotype (median: 0.0%, range: 0%–10% *vs*. median; 30.0%, range: 0%–100%, *P*=0.014) *vs*. non-carriers.

Discussion

In our study, VDR expression was predominantly assessed in cytoplasm of melanoma cells (79.7%, 59/74 cases), with few tumors (14.9%, 11/74 cases) displaying VDR positivity in the nucleus. Such findings partially contrast those of other studies performed in a Polish cohort of 69 patients with primary cutaneous melanoma (comprising 35 metastatic melanomas and that were classified as follows: 4 Clark I, 6 Clark II, 23 Clark III, 24 Clark IV, and 12 Clark V stage, where 30 were superficial-spreading, 37 were nodular, and two were acral lentiginous melanomas); these previous studies indicated VDR-positive nuclear immunostaining in 84.1% and cytoplasmic immunostaining in 66.7% of patients[38,39]. The study by Brożyna et al.[39], however, showed percentage of melanoma specimens with high nuclear staining at 17.4% (12/69), which is close to the percentage of nuclear staining in our study. Discrepancies between results of Brożyna et al. and our study probably arise from different antibodies employed[21], diverse histological characteristics, and/or geographical/genetic backgrounds of melanomas in the respective studies. According to European cancer observatory data[3], the estimated age standardized (European) incidence rate (per 100,000/year) of malignant cutaneous melanoma and mortality are 5.6 and 2.8 in Poland and 13.4 and 2.0 in Italy, respectively.

We further analyzed data regarding cytoplasmic VDR immunohistochemical staining. Once the cytoplasmic VDR binds with 1α, 25-dihydroxyvitamin D ligand, and adequate coreceptor protein, retinoid X receptor, it translocates to the nucleus, and by recruitment of coactivators and corepressors modulates transcription of target genes that encode proteins responsible for final activities induced by vitamin D hormonal signaling[18]. Consequently, absence or down-regulation of VDR expression may present implications for vitamin D resistance in melanoma tissues[38,48,49] and potentially modulates effects of vitamin D supplementation on prevention therapy of melanoma patients[49,50].

We did not observe effects of age at melanoma diagnosis and gender on VDR expression in cutaneous melanomas[45]. Roles of BMI and smoking were focuses of previous melanoma research[8,25,51]. In the present investigation, we did not observe the effects of BMI and smoking on VDR expression.

Consistent with studies performed by other authors on a Polish cohort[38,39], we observed that stage I melanomas were more frequent in tumors with high than with low VDR expression. In our study, stage IIA invasion was particularly more frequent in melanomas lacking VDR expression than in melanomas showing VDR expression. However, we did not observe significant data in relation to metastatic stages III and IV. Causes of these findings will require further enlarged studies.

Overall, metastatic melanomas did not exhibit different VDR expression from non-metastatic melanomas. Such results agreed with observations of some studies[13,20,48] but contradicted the findings of other authors[38,39]. Specific geographical/ethnic backgrounds possibly affected results. Thus, enlarged studies in subjects with different ethnicities should be performed in the future to substantiate this issue.

In our study, localization of melanoma on the body was unrelated with VDR expression. We only observed a tendency (P=0.093) for upper limb melanomas to be more frequent in tumors lacking VDR expression. Thus, despite the expected different exposures to sunlight and environmental factors of different parts of the human body, VDR expression in our study is not associated with body regions in which primary melanomas develop.

Lesion-specific characteristics did not correlate with VDR expression; these characteristics include ulceration, number of mitotic figures, regression, absence of TILs, non-brisk or brisk TILs, tumor emboli, and melanoma subtype (epithelioid and small cell). At variance, in Polish patients studied by Brożyna and colleagues[39] cytoplasmic VDR immunostaining was higher in group of brisk TIL-positive *vs*. that of absent and non-brisk TIL melanomas (P=0.01), and VDR expression was lower in melanomas with ulceration. We noted a tendency for higher frequency of superficial spreading (P=0.063) in melanomas with high than low VDR expression. Similarly, Brożyna and colleagues[38] observed higher VDR expression in superficially spreading than nodular melanomas.

Remarkably, VDR expression was related to tumor Breslow thickness and Clark levels in our Italian patients. Melanomas with a thickness below 1.00 mm were more frequently observed in cases with high than low VDR expression, whereas those with thickness of over or equal to 1.01 mm were more frequent in melanomas with low VDR expression. Clark level II (none of the studied melanomas presented a Clark I) was detected more frequently in melanomas with high than low VDR expression (P=0.025). Overall, such findings concur with previous data on Polish patients[38,39].

We observed that VDR expression was unrelated with the presence of multiple melanomas, additional non-melanoma skin cancers and non-skin cancers, and melanoma familiarity. To our knowledge, no previous study assessed these issues.

To our knowledge, our study was the first to investigate the relationship between VDR expression of human melanoma cells in excised tissues of patients and *VDR* polymorphisms. Out of four *VDR* polymorphisms investigated in our study, individual SNPs of FokI, BsmI, and TaqI did not display any relation with expression of VDR in melanoma. Only the ApaI genotype was correlated to VDR expression in melanoma. The heterozygous genotype Aa was identified in 61.1% of melanomas with high VDR expression *vs*. 36.8% of melanomas with low VDR expression (OR=2.69, P=0.037). A allele was more frequent in 100% than <100% VDR-positive cells (OR=3.16, P=0.038). To our knowledge, no research studied the role of *VDR*-ApaI polymorphism in VDR expression in melanoma tissues. *VDR*-ApaI SNP is located in an intron sequence and thus cannot directly modify the amino acid sequence of VDR protein; however, it participates in VDR RNA processing[26]. Recent evidence demonstrated that intronic sites of the VDR gene can function as binding sites of transcriptional regulators, such as p53[52,53]. A meta-analysis study[35] indicated that *VDR*-ApaI polymorphism of the European population features an association with overall skin cancer risk (Aa *vs*. AA, OR=1.27, CI=1.05–1.53; Aa+aa *vs*. AA, OR=1.23, CI=1.04–1.47). In a recent Italian study, the Aa heterozygous genotype was associated with increased risk of lumbar pathologies, especially osteochondrosis[29].

A number of combined genotypes in our study yielded significant findings according to VDR expression. The AaTT combined genotype was more frequent in melanomas with high than low VDR expression. Combined genotypes BbAA, bbAa, AATt, BbAATt, and bbAaTT were more frequent in 100% VDR-positive cells than <100% VDR-positive cells. The AATT combined genotype was much more frequent in subjects without VDR expression (20%) than in those with VDR expression (1.7%) (OR=14.5, P=0.025). No previous study investigated the relationship of VDR expression with *VDR* combined genotypes. Lack of VDR expression in excised melanoma tissues has been associated with reduced overall survival of patients[38,39]. Therefore, melanoma prognosis may be influenced by carrying a *VDR* combined genotype associated to absent or reduced VDR expression. In our melanoma patients, AATT is a rare combined genotype with a frequency of 5.4%, which is similar to the recent finding in an Italian cohort of 518 non-oncological subjects (6.0%)[29]. Further enlarged studies are warranted to assess roles of ApaI, BsmI, and TaqI combined genotypes in VDR expression in melanomas and their prognosis.

Regulation of VDR abundance is an important modulation mechanism of cellular responsiveness to 1α, 25-dihydroxyvitamin D[10]. Mechanisms underlying regulation of VDR abundance include alterations in transcription rate of VDR gene and/or stability of VDR mRNA and epigenetic changes[14,18,54]. Interestingly, treatment with calcitriol can

enhance VDR mRNA in cultured melanoma cells, showing that increasing vitamin D consumption can induce VDR expression[14]. On the other hand, enhanced melanogenesis was associated with downregulation of VDR expression[38,55,56]. Response of melanoma cells to calcitriol corresponds to expression level of VDR mRNA, which in turn may be regulated by VDR miRNAs and by epigenetically modulating drugs[50]. Remarkably, recent evidence suggests that tumor suppressors, such as p53, are implicated on direct regulation of VDR[53]. Molecules other than vitamin D, such as curcumin and vitamin E derivatives, were indicated as novel VDR ligands[19,57], whereas vitamin A derivatives were suggested as modulators of VDR actions[58]. Given the wide variety of positive and negative VDR modulators, each individual expression of VDR is highly dynamic in cells, with nuclear translocation of VDR fluctuating upon instant induction[18,19,22]. We speculate that unresponsiveness of some cutaneous melanomas to anti-proliferative effects of vitamin D possibly resulted from absence or insufficient VDR expression in melanoma and/or melanocytic cells. Further human studies are warranted to assess whether benefits of vitamin D augmentation are modulated by VDR expression in melanoma/melanocytic cells and/or by carriage of a specific *VDR* genotype and/or combined genotype polymorphisms that affect VDR expression. Such factors can modulate dose requirement of vitamin D treatments[59].

Roles of vitamin D in skin cancers still require complete elucidation[10,57]. All vitamin D actions virtually occur through VDR activation. Recent evidence shows that the effects of 1α, 25-dihydroxyvitamin D and VDR are mediated at least in part by cellular calcium levels; thus, calcium possibly contributes to the suppressive ability of VDR on skin cancer[10,57]. Deletion of VDR notably results in an increased susceptibility to tumorigenesis and also reduces ability of keratinocytes to clear UVB-induced DNA mutations[57]. VDR can bind to thousands of VDREs on human genome and up- or downregulate hundreds of genes[10,30,57]. Based on bioinformatic analysis, almost 15,000 sites in human DNA are bound by VDR, and 16%–21% of these putative binding sites are found at gene promoters[10,57,60]. Aside from classical VDRE-mediated mechanisms, increasing evidence point to regulatory contribution of several miRNAs[14,18,54] and long non-coding RNAs[10,57]. A recent study on VDR cistrome demonstrated the unexpected complexity of gene regulation, which was examined on a genome-wide scale in target tissues and cells, including a cross-talk between VDR and immune factors[60,61]. VDR expression is also commonly and significantly down regulated in colon adenocarcinoma. VDR cistrome analyses suggested that reduced VDR expression in colon cancer changes VDR activity by dampening expression of tumor suppressors by either stabilizing or inhibiting down regulation of oncogene expression. In turn, these effects may be associated with severe patient outcomes[62]. Thus, further research should study complex gene interactions and biological pathways related to vitamin D and melanoma and combine clinical evidence with molecular findings to support further progress[57,60,61].

Limitations of our study include the limited number of patients and absence of data on circulating vitamin D levels in patients at the time of melanoma excision. Strengths of this research comprise genetic background restrictions of enrolled patients and determination of demographic, lifestyle, histological, and genetic characteristics.

Given the sample size and multiple comparisons of our study, a validation of independent datasets with larger samples, and multivariable analysis will be necessary to adjust genetic traits for age, sex, tumor location, sun exposure, and smoking. Serum sampling in future studies may also be performed to determine circulating levels of vitamin D at time of melanoma excision.

Conclusions

Current information insufficiently discusses influence of vitamin D oral supplementation to direct VDR modulatory effects in human skin cells, including melanocytes and keratinocytes. Our present findings support the necessity of further studies on this issue by combining clinical and molecular approach.

Our study showed that VDR expression is associated with prognostic parameters of tumor Breslow thickness and Clark level. However, VDR expression was not related to metastatic melanomas. Our immunohistochemical results concur with those of a previous study on VDR expression in colorectal cancer, showing that VDR was not associated with tumor location, stage, and grade[42], and with another lung tumor study which demonstrated high variability of VDR expression[43]. Interestingly, we observed correlation between the Aa genotype and AaTT combined genotype with higher level of VDR expression and between AATT combined genotype and low or absent VDR expression; this new finding will require further validation. Future studies should assess whether VDR expression and *VDR* combined genotypes affect benefits of vitamin D and can drive an appropriate dose and schedule of calcitriol or other active (low calcemic) vitamin D analogs for melanoma treatment. Future set up of personalized nutrition and behavioral interventions will benefit from molecular studies exploring the connection of

biological pathways to bioactive components of food and cancer[63]. Our study suggests that determination of VDR expression in excised tissues of melanoma and/or determination of *VDR* genotypes carriage can be used as personalized tool of precision medicine when considering melanoma patients.

Acknowledgments

The authors thank Patrizia Nacci, Martina Linussio, Luca Bazzichetto and Silvia Lolini for their technical assistance.

Conflict of interest statement

No potential conflicts of interest are disclosed.

References

1. GLOBOCAN 2012: Estimated cancer incidence, mortality and prevalence worldwide in 2012. World Health Organization. International Agency for Research on Cancer (IARC). Available at: http://globocan.iarc.fr/Default.aspx; accessed March 27, 2017.
2. Arnold M, Holterhues C, Hollestein LM, Coebergh JWW, Nijsten T, Pukkala E, et al. Trends in incidence and predictions of cutaneous melanoma across Europe up to 2015. J Eur Acad Dermatol Venereol. 2014; 28: 1170-8.
3. EUCAN. Malignant melanoma of skin. Available at: http://eu-cancer.iarc.fr/EUCAN/Cancer.aspx?Cancer=20; accessed March 27, 2017.
4. Vos T, Barber RM, Bell B, Bertozzi-Villa A, Biryukov S, Bolliger I, et al. Global, regional, and national incidence, prevalence, and years lived with disability for 301 acute and chronic diseases and injuries in 188 countries, 1990-2013: a systematic analysis for the Global Burden of Disease Study 2013. Lancet. 2015; 386: 743-800.
5. ITACAN. AIRTUM. The Italian Association of Cancer Registries. I numeri del cancro in Italia - 2016. Available at: htttp://www.registri-tumori.it/itacan; accessed March 27, 2017.
6. Registro Tumori del Friuli Venezia Giulia. 2010: www.cro.sanita.fvg.it. accessed March 27, 2017.
7. Cecconi L, Busolin A, Barbone F, Serraino D, Chiarugi A, Biggeri A, et al. Spatial analysis of incidence of cutaneous melanoma in the Friuli Venezia Giulia region in the period 1995-2005. Geospat Health. 2016; 11: 422
8. Newton-Bishop JA, Davies JR, Latheef F, Randerson-Moor J, Chan M, Gascoyne J, et al. 25-Hydroxyvitamin D_2/D_3 levels and factors associated with systemic inflammation and melanoma survival in the Leeds melanoma Cohort. Int J Cancer. 2015; 136: 2890-9.
9. Pasquali S, Chiarion-Sileni V, Rossi CR, Mocellin S. Immune checkpoint inhibitors and targeted therapies for metastatic melanoma: A network meta-analysis. Cancer Treat Rev. 2017; 54: 34-42.
10. Bikle DD. Vitamin D receptor, a tumor suppressor in skin. Can J Physiol Pharmacol. 2015; 93: 349-54.
11. Evans SRT, Houghton AM, Schumaker L, Brenner RV, Buras RR, Davoodi F, et al. Vitamin D receptor and growth inhibition by 1,25-dihydroxyvitamin D_3 in human malignant melanoma cell lines. J Surg Res. 1996; 61: 127-33.
12. Trump DL, Deeb KK, Johnson CS. Vitamin D: considerations in the continued development as an agent for cancer prevention and therapy. Cancer J. 2010; 16: 1-9.
13. Danielsson C, Fehsel K, Polly P, Carlberg C. Differential apoptotic response of human melanoma cells to 1α,25-dihydroxyvitamin D_3 and its analogues. Cell Death Differ. 1998; 5: 946-52.
14. Seifert M, Rech M, Meineke V, Tilgen W, Reichrath J. Differential biological effects of 1,25-dihydroxyvitamin D_3 on melanoma cell lines in vitro. J Steroid Biochem Mol Biol. 2004; 89-90: 375-9.
15. Feldman D, Krishnan AV, Swami S, Giovannucci E, Feldman BJ. The role of vitamin D in reducing cancer risk and progression. Nat Rev Cancer. 2014; 14: 342-57.
16. Holick MF. Biological effects of sunlight, ultraviolet radiation, visible light, infrared radiation and vitamin D for health. Anticancer Res. 2016; 36: 1345-56.
17. Sehdev A, O'Neil BH. The role of aspirin, vitamin D, exercise, diet, statins, and metformin in the prevention and treatment of colorectal cancer. Curr Treat Options Oncol. 2015; 16: 43
18. Dou RX, Ng K, Giovannucci EL, Manson JE, Qian ZR, Ogino S. Vitamin D and colorectal cancer: molecular, epidemiological and clinical evidence. Br J Nutr. 2016; 115: 1643-60.
19. Haussler MR, Whitfield GK, Kaneko I, Haussler CA, Hsieh D, Hsieh JC, et al. Molecular mechanisms of vitamin D action. Calcif Tissue Int. 2013; 92: 77-98.
20. Keum N, Giovannucci E. Vitamin D supplements and cancer incidence and mortality: a meta-analysis. Br J Cancer. 2014; 111: 976-80.
21. Bikle DD. Vitamin D metabolism, mechanism of action, and clinical applications. Chem Biol. 2014; 21: 319-29.
22. Lee SM, Pike JW. The vitamin D receptor functions as a transcription regulator in the absence of 1,25-dihydroxyvitamin D_3. J Steroid Biochem Mol Biol. 2016; 164: 265-70.
23. Colston K, Colston MJ, Feldman D. 1,25-dihydroxyvitamin D_3 and malignant melanoma: the presence of receptors and inhibition of cell growth in culture. Endocrinology. 1981; 108: 1083-6.
24. Bonin S, Albano A, di Meo N, Gatti A, Stinco G, Zanconati F, et al. Cutaneous melanoma frequencies and seasonal trend in 20 years of observation of a population characterised by excessive sun exposure. Radiol Oncol. 2015; 49: 379-85.
25. Gandini S, Montella M, Ayala F, Benedetto L, Rossi CR, Vecchiato A, et al. Sun exposure and melanoma prognostic factors. Oncol Lett. 2016; 11: 2706-14.
26. Uitterlinden AG, Fang Y, Van Meurs JBJ, Pols HAP, Van Leeuwen JPTM. Genetics and biology of vitamin D receptor polymorphisms. Gene. 2004; 338: 143-56.
27. Selvaraj P, Chandra G, Jawahar MS, Rani MV, Rajeshwari DN, Narayanan PR. Regulatory role of vitamin D receptor gene variants

of *Bsm*I, *Apa*I, *Taq*I, and *Fok*I polymorphisms on macrophage phagocytosis and lymphoproliferative response to *Mycobacterium tuberculosis* antigen in pulmonary tuberculosis. J Clin Immunol. 2004; 24: 523-32.

28. Colombini A, Cauci S, Lombardi G, Lanteri P, Croiset S, Brayda-Bruno M, et al. Relationship between vitamin D receptor gene (*VDR*) polymorphisms, vitamin D status, osteoarthritis and intervertebral disc degeneration. J Steroid Biochem Mol Biol. 2013; 138: 24-40.
29. Colombini A, Brayda-Bruno M, Lombardi G, Croiset SJ, Ceriani C, Buligan C, et al. *Bsm*I, *Apa*I and *Taq*I polymorphisms in the vitamin D receptor gene (*VDR*) and association with lumbar spine pathologies: An Italian case-control study. PLoS One. 2016; 11: e0155004
30. Gandini S, Gnagnarella P, Serrano D, Pasquali E, Raimondi S. Vitamin D receptor polymorphisms and cancer. Adv Exp Med Biol. 2014; 810: 69-105.
31. Orlow I, Reiner AS, Thomas NE, Roy P, Kanetsky PA, Luo L, et al. Vitamin D receptor polymorphisms and survival in patients with cutaneous melanoma: a population-based study. Carcinogenesis. 2016; 37: 30-8.
32. Santonocito C, Capizzi R, Concolino P, Lavieri MM, Paradisi A, Gentileschi S, et al. Association between cutaneous melanoma, Breslow thickness and vitamin D receptor *Bsm*I polymorphism. Br J Dermatol. 2007; 156: 277-82.
33. Randerson-Moor JA, Taylor JC, Elliott F, Chang YM, Beswick S, Kukalizch K, et al. Vitamin D receptor gene polymorphisms, serum 25-hydroxyvitamin D levels, and melanoma: UK case-control comparisons and a meta-analysis of published VDR data. Eur J Cancer. 2009; 45: 3271-81.
34. Mandelcorn-Monson R, Marrett L, Kricker A, Armstrong BK, Orlow I, Goumas C, et al. Sun exposure, vitamin D receptor polymorphisms *Fok*I and *Bsm*I and risk of multiple primary melanoma. Cancer Epidemiol. 2011; 35: e105-10.
35. Zhao XZ, Yang BH, Yu GH, Liu SZ, Yuan ZY. Polymorphisms in the vitamin D receptor (VDR) genes and skin cancer risk in European population: a meta-analysis. Arch Dermatol Res. 2014; 306: 545-53.
36. Lee YH, Gyu Song G. Vitamin D receptor *Fok*I, *Bsm*I, *Taq*I, *Apa*I, and *Eco*RV polymorphisms and susceptibility to melanoma: a meta-analysis. J BUON. 2015; 20: 235-43.
37. Hou W, Wan XF, Fan JW. Variants Fok1 and Bsm1 on VDR are associated with the melanoma risk: evidence from the published epidemiological studies. BMC Genet. 2015; 16: 14
38. Brożyna AA, Jozwicki W, Janjetovic Z, Slominski AT. Expression of vitamin D receptor decreases during progression of pigmented skin lesions. Hum Pathol. 2011; 42: 618-31.
39. Brożyna AA, Jóźwicki W, Slominski AT. Decreased VDR expression in cutaneous melanomas as marker of tumor progression: new data and analyses. Anticancer Res. 2014; 34: 2735-43.
40. Erdmann F, Lortet-Tieulent J, Schüz J, Zeeb H, Greinert R, Breitbart EW, et al. International trends in the incidence of malignant melanoma 1953-2008 - are recent generations at higher or lower risk? Int J Cancer. 2013; 132: 385-400.
41. Balch CM, Gershenwald JE, Soong SJ, Thompson JF, Atkins MB, Byrd DR, et al. Final version of 2009 AJCC melanoma staging and classification. J Clin Oncol. 2009; 27: 6199-206.
42. Kure S, Nosho K, Baba Y, Irahara N, Shima K, Ng K, et al. Vitamin D receptor expression is associated with *PIK3CA* and *KRAS* mutations in colorectal cancer. Cancer Epidemiol Biomarkers Prev. 2009; 18: 2765-72.
43. Turner AM, McGowan L, Millen A, Rajesh P, Webster C, Langman G, et al. Circulating DBP level and prognosis in operated lung cancer: an exploration of pathophysiology. Eur Respir J. 2013; 41: 410-16.
44. Di Loreto C, La Marra F, Mazzon G, Belgrano E, Trombetta C, Cauci S. Immunohistochemical evaluation of androgen receptor and nerve structure density in human prepuce from patients with persistent sexual side effects after finasteride use for androgenetic alopecia. PLoS One. 2014; 9: e0100237
45. Colombini A, Brayda-Bruno M, Ferino L, Lombardi G, Maione V, Banfi G, et al. Gender differences in the *VDR*-FokI polymorphism and conventional non-genetic risk factors in association with lumbar spine pathologies in an Italian case-control study. Int J Mol Sci. 2015; 16: 3722-39.
46. Colombini A, Brayda-Bruno M, Lombardi G, Croiset SJ, Vrech V, Maione V, et al. FokI polymorphism in the vitamin D receptor gene (*VDR*) and its association with lumbar spine pathologies in the Italian population: a case-control study. PLoS One. 2014; 9: e97027
47. Cauci S, Migliozzi F, Trombetta CS, Venuto I, Saccheri P, Travan L, et al. Low back pain and FokI (rs2228570) polymorphism of vitamin D receptor in athletes. BMC Sports Sci Med Rehabil. 2017; 9: 4
48. Reichrath J, Rech M, Moeini M, Meese E, Tilgen W, Seifert M. In vitro comparison of the vitamin D endocrine system in 1,25(OH)2D3-responsive and resistant melanoma cells. Cancer Biol Ther. 2007; 6: 48-55.
49. Pervin S, Hewison M, Braga M, Tran L, Chun R, Karam A, et al. Down-regulation of vitamin D receptor in mammospheres: implications for vitamin D resistance in breast cancer and potential for combination therapy. PLoS One. 2013; 8: e53287
50. Essa S, Reichrath S, Mahlknecht U, Montenarh M, Vogt T, Reichrath J. Signature of VDR miRNAs and epigenetic modulation of vitamin D signaling in melanoma cell lines. Anticancer Res. 2012; 32: 383-9.
51. Jiang AJ, Rambhatla PV, Eide MJ. Socioeconomic and lifestyle factors and melanoma: a systematic review. Br J Dermatol. 2015; 172: 885-915.
52. Maruyama R, Aoki F, Toyota M, Sasaki Y, Akashi H, Mita H, et al. Comparative genome analysis identifies the vitamin D receptor gene as a direct target of p53-mediated transcriptional activation. Cancer Res. 2006; 66: 4574-83.
53. Reichrath J, Reichrath S, Heyne K, Vogt T, Roemer K. Tumor suppression in skin and other tissues via cross-talk between vitamin D- and p53-signaling. Front Physiol. 2014; 5: 166

54. Essa S, Denzer N, Mahlknecht U, Klein R, Collnot EM, Tilgen W, et al. VDR microRNA expression and epigenetic silencing of vitamin D signaling in melanoma cells. J Steroid Biochem Mol Biol. 2010; 121: 110-3.
55. Janjetovic Z, Brozyna AA, Tuckey RC, Kim TK, Nguyen MN, Jozwicki W, et al. High basal NF-κB activity in nonpigmented melanoma cells is associated with an enhanced sensitivity to vitamin D_3 derivatives. Br J Cancer. 2011; 105: 1874-84.
56. Wasiewicz T, Szyszka P, Cichorek M, Janjetovic Z, Tuckey RC, Slominski AT, et al. Antitumor effects of vitamin D analogs on hamster and mouse melanoma cell lines in relation to melanin pigmentation. Int J Mol Sci. 2015; 16: 6645-67.
57. Bikle DD, Oda Y, Tu CL, Jiang Y. Novel mechanisms for the vitamin D receptor (VDR) in the skin and in skin cancer. J Steroid Biochem Mol Biol. 2015; 148: 47-51.
58. Danielsson C, Törmä H, Vahlquist A, Carlberg C. Positive and negative interaction of 1,25-dihydroxyvitamin D3 and the retinoid CD437 in the induction of human melanoma cell apoptosis. Int J Cancer. 1999; 81: 467-70.
59. Bischoff-Ferrari HA, Shao A, Dawson-Hughes B, Hathcock J, Giovannucci E, Willett WC. Benefit-risk assessment of vitamin D supplementation. Osteoporos Int. 2010; 21: 1121-32.
60. Pike JW, Meyer MB, Lee SM, Onal M, Benkusky NA. The vitamin D receptor: contemporary genomic approaches reveal new basic and translational insights. J Clin Invest. 2017; 127: 1146-54.
61. Singh PK, van den Berg PR, Long MD, Vreugdenhil A, Grieshober L, Ochs-Balcom HM, et al. Integration of VDR genome wide binding and GWAS genetic variation data reveals co-occurrence of VDR and NF-κB binding that is linked to immune phenotypes. BMC Genomics. 2017; 18: 132
62. Long MD, Campbell MJ. Integrative genomic approaches to dissect clinically-significant relationships between the VDR cistrome and gene expression in primary colon cancer. J Steroid Biochem Mol Biol. 2016; doi: 10.1016/j.jsbmb.2016.12.013.
63. Rescigno T, Micolucci L, Tecce MF, Capasso A. Bioactive nutrients and nutrigenomics in age-related diseases. Molecules. 2017; 22: 105

Cite this article as: La Marra F, Stinco G, Buligan C, Chiriacò G, Serraino D, Di Loreto C, et al. Immunohistochemical evaluation of vitamin D receptor (VDR) expression in cutaneous melanoma tissues and four VDR gene polymorphisms. Cancer Biol Med. 2017; 14: 162-75. doi: 10.20892/j.issn.2095-3941.2017.0020

Table S1 Clinical characteristics of 74 consecutively enrolled melanoma patients and comparison between groups of 100% VDR-positive cells (*n*=12) *vs.* <100% (*n*=62); over the median (>20%) VDR-positive cell (*n*=36) *vs.* below or equal the median (≤20%) (*n*=38); and absence of VDR-positive cell (*n*=15) *vs.* remaining cases with detected VDR expression (*n*=59)

Characteristics	All melanoma patients (*n*=74)	100% VDR-positive cells (*n*=12)	<100% VDR-positive cells (*n*=62)	OR (CI)100% *vs.* <100% VDR-positive	*P*=100% *vs.* < 100% VDR-positive	>20%[a] VDR-positive cells (*n*=36)	≤20% VDR-positive cells (*n*=38)	OR (CI)>20% *vs.* ≤ 20% VDR-positive	*P*>20% *vs.* ≤ 20% VDR-positive	VDR absence (0% positive) (*n*=15)	VDR presence (>0% positive) (*n*=59)	OR (CI)VDR absence *vs.* presence	PVDR absence *vs.* presenc
Age at melanoma diagnosis, years, mean ± SD	52.1±12.7	49.8±13.0	52.7±12.9	–	0.519[a]	51.7±13.9	52.8±12.0	–	0.770[a]	51.5±14.5	52.5±12.6	–	0.767[a]
Age <50 years at melanoma diagnosis	31 (41.9)	6 (50.0)	25 (40.3)	1.48 (0.43–5.11)	0.534	16 (44.4)	15 (39.5)	1.23 (0.49–3.09)	0.665	7 (46.7)	24 (40.7)	1.28 (0.41–3.99)	0.675
BMI, kg/m^2, mean ± SD	25.8±4.0	25.0±4.6	25.9±3.9	–	0.412[a]	26.0±4.3	25.6±3.8	–	0.804[a]	25.7±2.6	25.8±4.3	–	0.762[a]
Male	39 (52.7)	6 (50.0)	33 (53.2)	0.88 (0.25–3.03)	0.838	20 (55.6)	19 (50.0)	1.25 (0.50–3.12)	0.632	9 (60.0)	30 (50.8)	1.45 (0.46–4.59)	0.526
Smoker	11 (14.9)	2 (16.7)	9 (14.5)	1.18 (0.22–6.28)	1.000	4 (11.1)	7 (18.4)	0.55 (0.15–2.08)	0.377	2 (13.3)	9 (15.3)	0.85 (0.16–4.45)	1.000
Stage I	38 (51.4)	6 (50.0)	32 (51.6)	0.94 (0.27–3.23)	0.919	23 (63.9)	15 (39.5)	2.71 (1.06–6.95)	0.036	6 (40.0)	32 (54.2)	0.56 (0.18–1.78)	0.325
Stage I A	23 (31.1)	4 (33.3)	19 (30.6)	1.13 (0.30–4.22)	1.000	15 (41.7)	8 (21.1)	2.68 (0.96–7.45)	0.055	3 (20.0)	20 (33.9)	0.49 (0.12–1.93)	0.365
Stage I B	15 (20.3)	2 (16.7)	13 (21.0)	0.75 (0.15–3.87)	1.000	8 (22.2)	7 (18.4)	1.26 (0.41–3.94)	0.684	3 (20.0)	12 (20.3)	0.98 (0.24–4.03)	1.000
Stage II	16 (21.6)	2 (16.7)	14 (22.6)	0.69 (0.13–3.50)	1.000	4 (11.1)	12 (31.6)	0.27 (0.08–0.94)	0.033	6 (40.0)	10 (16.9)	3.27 (0.95–11.3)	0.077
Stage II A	7 (9.5)	0 (–)	7 (11.3)	–[b]	0.590	0 (–)	7 (18.4)	–[b]	0.012	4 (26.7)	3 (5.1)	6.79 (1.33–34.7)	0.028
Stage II B	9 (12.2)	2 (16.7)	7 (11.3)	1.57 (0.28–8.69)	0.633	4 (11.1)	5 (13.2)	0.82 (0.20–3.35)	1.000	2 (13.3)	7 (11.9)	1.14 (0.21–6.16)	1.000
Stage II C	0 (–)	0 (–)	0 (–)	–[b]	–[b]	0 (–)	0 (–)	–[b]	–[b]	0 (–)	0 (–)	–[b]	–[b]
Stage III	10 (13.5)	3 (25.0)	7 (11.3)	2.62 (0.57–12.0)	0.351	6 (16.7)	4 (10.5)	1.70 (0.44–6.60)	0.510	1 (6.7)	9 (15.3)	0.40 (0.05–3.40)	0.676
Stage III A	4 (5.4)	1 (8.3)	3 (4.8)	1.79 (0.17–18.8)	0.515	3 (8.3)	1 (2.6)	3.36 (0.33–33.9)	0.351	0 (–)	4 (6.8)	–[b]	0.576
Stage III B	3 (4.1)	0 (–)	3 (4.8)	–[b]	1.000	0 (–)	3 (7.9)	–[b]	0.240	1 (6.7)	2 (3.4)	2.04 (0.17–24.1)	0.499
Stage III C	3 (4.1)	2 (16.7)	1 (1.6)	12.2 (1.01–147)	0.067	3 (8.3)	0 (–)	–[b]	0.110	0 (–)	3 (5.1)	–[b]	1.000

Continued

Continued

Characteristics	All melanoma patients (*n*=74)	100% VDR-positive cells (*n*=12)	<100% VDR-positive cells (*n*=62)	OR (CI)100% *vs.* <100% VDR-positive	*P*=100% *vs.* < 100% VDR-positive	>20%[a] VDR-positive cells (*n*=36)	≤20% VDR-positive cells (*n*=38)	OR (CI)>20% *vs.* ≤ 20% VDR-positive	*P*>20% *vs.* ≤ 20% VDR-positive	VDR absence (0% positive) (*n*=15)	VDR presence (>0% positive) (*n*=59)	OR (CI)VDR absence *vs.* presence	PVDR absence *vs.* presence
Stage IV	10 (13.5)	1 (8.3)	9 (14.5)	0.53 (0.06–4.67)	1.000	3 (8.3)	7 (18.4)	0.40 (0.10–1.70)	0.310	2 (13.3)	8 (13.6)	0.98 (0.19–5.18)	1.000
Metastatic melanoma (Stages III +IV)	20 (27.0)	4 (33.3)	16 (25.8)	1.44 (0.38–5.43)	0.724	9 (25.0)	11 (28.9)	0.82 (0.29–2.29)	0.702	3 (20.0)	17 (28.8)	0.62 (0.15–2.47)	0.746
Trunk	47 (63.5)	5 (41.7)	42 (67.7)	0.34 (0.10–1.20)	0.108	24 (66.7)	23 (60.5)	1.30 (0.50–3.37)	0.583	7 (46.7)	40 (67.8)	0.42 (0.13–1.31)	0.129
Upper limb	6 (8.1)	2 (16.7)	4 (6.5)	2.90 (0.47–18.0)	0.249	2 (5.6)	4 (10.5)	0.50 (0.09–2.91)	0.675	3 (20.0)	3 (5.1)	4.67 (0.84–26.0)	0.093
Lower limb	14 (18.9)	3 (25.0)	11 (17.7)	1.54 (0.36–6.65)	0.687	7 (19.4)	7 (18.4)	1.07 (0.33–3.42)	0.911	4 (26.7)	10 (16.9)	1.78 (0.47–6.74)	0.463
Hands/feet	5 (6.8)	2 (16.7)	3 (4.8)	3.93 (0.58–26.6)	0.183	2 (5.6)	3 (7.9)	0.69 (0.11–4.37)	1.000	0 (–)	5 (8.5)	–[b]	0.576
Head/neck	2 (2.7)	0 (–)	2 (3.2)	–[b]	1.000	1 (2.8)	1 (2.6)	1.06 (0.06–17.6)	1.000	1 (6.7)	1 (1.7)	4.14 (0.24–70.4)	0.367
Superficial spreading	37 (50.0)	7 (58.3)	30 (48.4)	1.49 (0.43–5.22)	0.528	22 (61.1)	15 (39.5)	2.41 (0.95–6.13)	0.063	6 (40.0)	31 (52.5)	0.60 (0.19–1.91)	0.386
Nodular	31 (41.9)	4 (33.3)	27 (43.5)	0.65 (0.18–2.38)	0.512	12 (33.3)	19 (50.0)	0.50 (0.19–1.28)	0.146	8 (53.3)	23 (39.0)	1.79 (0.57–5.60)	0.314
Acral lentiginous	3 (4.1)	1 (8.3)	2 (3.2)	2.73 (0.23–32.7)	0.417	1 (2.8)	2 (5.3)	0.51 (0.04–5.93)	1.000	0 (–)	3 (5.1)	–[b]	1.000
Spitzoide	2 (2.7)	0 (–)	2 (3.2)	–[b]	1.000	1 (2.8)	1 (2.6)	1.06 (0.06–17.6)	1.000	1 (6.7)	1 (1.7)	4.14 (0.24–70.4)	0.367
Others	3 (4.1)	0 (–)	3 (4.8)	–[b]	1.000	0 (–)	3 (7.9)	–[b]	0.240	0 (–)	3 (5.1)	–[b]	1.000
Breslow thickness, mm, mean ± SD	1.77±1.62	1.34±0.94	1.85±1.71	–	0.416[a]	1.23±0.88	2.27±1.97	–	0.008[a]	2.16±1.88	1.66±1.55	–	0.145[a]
Breslow thickness <1.00 mm	28 (37.8)	4 (33.3)	24 (38.7)	0.79 (0.21–2.92)	1.000	18 (50.0)	10 (26.3)	2.80 (1.06–7.41)	0.036	4 (26.7)	24 (40.7)	0.53 (0.15–1.86)	0.318
Breslow thickness ≥1.01 mm	46 (62.2)	8 (66.7)	38 (61.3)	1.26 (0.34–4.66)	1.000	18 (50.0)	28 (77.8)	0.36 (0.13–0.95)	0.036	11 (73.3)	35 (59.3)	1.89 (0.54–6.63)	0.318
Clark I	0 (–)	0 (–)	0 (–)	–[b]		0 (–)	0 (–)	–[b]	–[b]	0 (–)	0 (–)	–[b]	–[b]
Clark II	20 (27.0)	5 (41.7)	15 (24.2)	2.24 (0.62–8.10)	0.287	14 (38.9)	6 (15.8)	3.39 (1.13–10.2)	0.025	2 (13.3)	18 (30.5)	0.35 (0.07–1.72)	0.328

Continued

Continued

Characteristics	All melanoma patients (*n*=74)	100% VDR-positive cells (*n*=12)	<100% VDR-positive cells (*n*=62)	OR (CI)100% *vs.* <100% VDR-positive	*P*=100% *vs.* < 100% VDR-positive	>20%[a] VDR-positive cells (*n*=36)	≤20% VDR-positive cells (*n*=38)	OR (CI)>20% *vs.* ≤ 20% VDR-positive	*P*>20% *vs.* ≤ 20% VDR-positive	VDR absence (0% positive) (*n*=15)	VDR presence (>0% positive) (*n*=59)	OR (CI)VDR absence *vs.* presence	PVDR absence *vs.* presence
Clark III	15 (20.3)	1 (8.3)	14 (22.6)	0.31 (0.04–2.63)	0.439	6 (16.7)	9 (23.7)	0.64 (0.20–2.04)	0.453	4 (26.7)	11 (18.6)	1.59 (0.42–5.93)	0.488
Clark IV	36 (48.6)	6 (50.0)	30 (48.4)	1.07 (0.31–3.67)	0.919	15 (41.7)	21 (55.3)	0.58 (0.23–1.45)	0.242	8 (53.3)	28 (47.5)	1.26 (0.41–3.94)	0.684
Clark V	2 (2.7)	0 (–)	2 (3.2)	–[b]	1.000	0 (–)	2 (5.3)	–[b]	0.494	1 (6.7)	1 (1.7)	4.14 (0.24–70.4)	0.367
Ulceration	25 (33.8)	5 (41.7)	20 (32.3)	1.50 (0.42–5.31)	0.525	11 (30.6)	14 (36.8)	0.75 (0.29–1.99)	0.568	5 (33.3)	20 (33.9)	0.97 (0.29–3.24)	0.967
Mitosis >1	51 (68.9)	8 (66.7)	43 (69.4)	0.88 (0.24–3.29)	1.000	23 (63.9)	28 (73.7)	0.63 (0.23–1.70)	0.363	12 (80.0)	39 (66.1)	2.05 (0.52–8.11)	0.365
Regression	12 (16.2)	1 (8.3)	11 (17.7)	0.42 (0.05–3.61)	0.677	6 (16.7)	6 (15.8)	1.07 (0.31–3.67)	0.919	4 (26.7)	8 (13.6)	2.32 (0.59–9.08)	0.248
Brisk positive TILs[a]	25 (33.8)	5 (41.7)	20 (32.3)	1.50 (0.42–5.31)	0.525	15 (41.7)	10 (26.3)	2.00 (0.75–5.33)	0.163	5 (33.3)	20 (33.9)	0.97 (0.29–3.24)	0.967
Non-brisk TILs[a]	26 (35.1)	3 (25.0)	23 (37.1)	0.56 (0.14–2.30)	0.522	10 (27.8)	16 (42.1)	0.53 (0.20–1.40)	0.197	7 (46.7)	19 (32.2)	1.84 (0.58–5.83)	0.295
TILs absence[a]	23 (31.1)	4 (33.3)	19 (30.6)	1.13 (0.30–4.22)	1.000	11 (30.6)	12 (31.6)	0.95 (0.36–2.55)	0.924	3 (20.0)	20 (33.9)	0.49 (0.12–1.93)	0.365
Emboli	9 (12.2)	2 (16.7)	7 (11.3)	1.57 (0.28–8.69)	0.633	4 (11.1)	5 (13.2)	0.82 (0.20–3.35)	1.000	2 (13.3)	7 (11.9)	1.14 (0.21–6.16)	1.000
Microsatellitosis	2 (2.7)	0 (–)	2 (3.2)	–[b]		1 (2.8)	1 (2.6)	1.06 (0.06–17.6)	1.000	0 (–)	2 (3.4)	–[b]	1.000
Epithelioid variant	11 (14.9)	3 (25.0)	8 (12.9)	2.25 (0.50–10.1)	0.371	6 (16.7)	5 (13.2)	1.32 (0.36–4.77)	0.672	0 (–)	11 (18.6)	–[b]	0.106
Fusate variant	4 (5.4)	0 (–)	4 (6.5)	–[b]	1.000	3 (8.3)	1 (2.6)	3.36 (0.33–33.9)	0.351	0 (–)	4 (6.8)	–[b]	0.576
Small-cell variant	2 (2.7)	0 (–)	2 (3.2)	–[b]	1.000	1 (2.8)	1 (2.6)	1.06 (0.06–17.6)	1.000	1 (6.7)	1 (1.7)	4.14 (0.24–70.4)	0.367
>1 melanoma	9 (12.2)	1 (8.3)	8 (12.9)	0.61 (0.07–5.41)	1.000	4 (11.1)	5 (13.2)	0.82 (0.20–3.35)	1.000	2 (13.3)	7 (11.9)	1.14 (0.21–6.16)	1.000
Additional non-melanoma skin cancer	11 (14.9)	2 (16.7)	9 (14.5)	1.18 (0.22–6.28)	1.000	6 (16.7)	5 (13.2)	0.32 (0.36–4.77)	0.672	3 (20.0)	8 (13.6)	1.59 (0.37–6.92)	0.684
Additional non-skin cancer	14 (18.9)	3 (25.0)	11 (17.7)	1.54 (0.36–6.65)	0.687	9 (25.0)	5 (13.2)	2.20 (0.66–7.35)	0.194	3 (20.0)	11 (18.6)	1.09 (0.26–4.53)	1.000
Melanoma familiarity	13 (17.6)	3 (25.0)	10 (16.1)	1.73 (0.40–7.55)	0.432	8 (22.2)	5 (13.2)	1.89 (0.55–6.42)	0.306	1 (6.7)	12 (20.3)	0.28 (0.03–2.34)	0.282

[a] Two-tailed Mann–Whitney U test. [b] OR uncountable because one or two of compared groups included zero subject. [c] TILs, tumor infiltrating lymphocytes.

Table S2 Genotype and allele of ApaI, VDR-polymorphism, and BsmI-ApaI, ApaI-TaqI, BsmI-ApaI-TaqI combined genotypes of 74 melanoma patients and comparisons between groups of 100% VDR-positive cells (*n*=12) *vs*. <100% (*n*=62); over the median (>20%) VDR-positive cells (*n*=36) *vs*. below or equal the median (≤20%) (*n*=38); and absence of VDR-positive cell (*n*=15) *vs*. remaining cases with detected VDR expression (*n*=59)

VDR genotype or combined genotype	All melanoma patients (*n*=74)	100% VDR-positive cells (*n*=12)	<100% VDR-positive cells (*n*=62)	OR (CI) 100% *vs*. <100% VDR-positive	*P*=100% *vs*. <100% VDR-positive	>20%[a] VDR-positive cells (*n*=36)	≤20% VDR-positive cells (*n*=38)	OR (CI)>20% *vs*. ≤20% VDR-positive	*P*>20% *vs*.≤20% VDR-positive	VDR absence (0% positive) (*n*=15)	VDR presence (>0% positive) (*n*=59)	OR (CI) VDR absencevs. presence	PVDR absence *vs*. presence
ApaI genotype													
AA	30 (40.5)	8 (66.7)	22 (35.5)	3.64 (0.98–13.5)	0.058	13 (36.1)	17 (44.7)	0.70 (0.27–1.78)	0.450	8 (53.3)	22 (37.3)	1.92 (0.61–6.03)	0.258
Aa	36 (48.6)	4 (33.3)	32 (51.6)	0.47 (0.13–1.72)	0.246	22 (61.1)	14 (36.8)	2.69 (1.05–6.90)	0.037	5 (33.3)	31 (52.5)	0.45 (0.14–1.48)	0.184
aa	8 (10.8)	0 (–)	8 (12.9)	–[b]	0.339	1 (2.8)	7 (18.4)	0.13 (0.01–1.09)	0.056	2 (13.3)	6 (10.2)	1.36 (0.24–7.52)	0.660
A allele	96/148 (64.9)	20/24 (83.3)	76/124 (61.3)	3.16 (1.02–9.80)	0.038	48/72 (66.7)	48/76 (63.2)	1.17 (0.59–2.29)	0.655	21/30 (70.0)	75/118 (63.6)	1.34 (0.56–3.18)	0.509
a allele	52/148 (35.1)	4/24 (16.7)	48/124 (38.7)	0.32 (0.10–0.98)	0.038	24/72 (33.3)	28/76 (36.8)	0.86 (0.44–1.69)	0.655	9/30 (30.0)	43/118 (36.4)	0.75 (0.31–1.78)	0.509
BsmI-ApaI combined genotype													
BBAA	23 (31.1)	5 (41.7)	18 (29.0)	1.75 (0.49–6.23)	0.498	9 (25.0)	14 (36.8)	0.57 (0.21–1.56)	0.271	6 (40.0)	17 (28.8)	1.65 (0.51–5.34)	0.533
BbAA	5 (6.8)	3 (25.0)	2 (3.2)	10.0 (1.46–68.3)	0.028	4 (11.1)	1 (2.6)	4.62 (0.49–43.5)	0.194	1 (6.7)	4 (6.8)	0.98 (0.10–9.49)	1.000
BbAa	31 (41.9)	1 (8.3)	30 (48.4)	0.10 (0.01–0.80)	0.010	18 (50.0)	13 (34.2)	1.92 (0.75–4.90)	0.169	5 (33.3)	26 (44.1)	0.63 (0.19–2.09)	0.452
Bbaa	2 (2.7)	0 (–)	2 (3.2)	–[b]	1.000	0 (–)	2 (5.3)	–[b]	0.494	0 (–)	2 (3.4)	–[b]	1.000
bbAA	2 (2.7)	0 (–)	2 (3.2)	–[b]	1.000	0 (–)	2 (5.3)	–[b]	0.494	1 (6.7)	1 (1.7)	4.14 (0.24–70.4)	0.367
bbAa	5 (6.8)	3 (25.0)	2 (3.2)	10.0 (1.46–68.3)	0.028	4 (11.1)	1 (2.6)	4.62 (0.49–43.5)	0.194	0 (–)	5 (8.5)	–[b]	0.576
bbaa	6 (8.1)	0 (–)	6 (9.7)	–[b]	0.581	1 (2.8)	5 (13.2)	0.19 (0.02–1.70)	0.200	2 (13.3)	4 (6.8)	2.11 (0.35–12.8)	0.595
ApaI-TaqI combined genotype													
AATT	4 (5.4)	0 (–)	4 (6.5)	–[b]	1.000	0 (–)	4 (10.5)	–[b]	0.115	3 (20.0)	1 (1.7)	14.5 (1.39–152)	0.025
AATt	14 (18.9)	6 (50.0)	8 (12.9)	6.75 (1.74–26.1)	0.008	9 (25.0)	5 (13.2)	2.20 (0.66–7.35)	0.194	4 (26.7)	10 (16.9)	1.78 (0.47–6.74)	0.463
AAtt	12 (16.2)	2 (16.7)	10 (16.1)	1.04 (0.20–5.48)	1.000	4 (11.1)	8 (21.1)	0.47 (0.13–1.72)	0.246	1 (6.7)	11 (18.6)	0.31 (0.04–2.63)	0.439
AaTT	12 (16.2)	3 (25.0)	9 (14.5)	1.96 (0.44–8.67)	0.399	9 (25.0)	3 (7.9)	3.89 (0.96–15.8)	0.046	1 (6.7)	11 (18.6)	0.31 (0.04–2.63)	0.439
AaTt	24 (32.4)	1 (8.3)	23 (37.1)	0.15 (0.02–1.27)	0.089^	13 (36.1)	11 (28.9)	1.39 (0.52–3.68)	0.511	4 (26.7)	20 (33.9)	0.71 (0.20–2.51)	0.761
aaTT	8 (10.8)	0 (–)	8 (12.9)	–[b]	0.339	1 (2.8)	7 (18.4)	0.13 (0.01–1.09)	0.056	2 (13.3)	6 (10.2)	1.36 (0.24–7.52)	0.660
BsmI-ApaI-TaqI combined genotype													
BBAATT	1 (1.4)	0 (–)	1 (1.6)	–[b]	1.000	0 (–)	1 (2.6)	–[b]	1.000	1 (6.7)	0 (–)	–[b]	0.203
BBAATt	10 (13.5)	3 (25.0)	7 (11.3)	2.62 (0.57–12.0)	0.351	5 (13.9)	5 (13.2)	1.06 (0.28–4.04)	1.000	4 (26.7)	6 (10.2)	3.21 (0.77–13.3)	0.110

Continued

Continued

VDR genotype or combined genotype	All melanoma patients (*n*=74)	100% VDR-positive cells (*n*=12)	<100% VDR-positive cells (*n*=62)	OR (CI) 100% *vs.* <100% VDR-positive	*P*=100% *vs.* <100% VDR-positive	>20%[a] VDR-positive cells (*n*=36)	≤20% VDR-positive cells (*n*=38)	OR (CI)>20% *vs.* ≤20% VDR-positive	*P*>20% *vs.*≤20% VDR-positive	VDR absence (0% positive) (*n*=15)	VDR presence (>0% positive) (*n*=59)	OR (CI) VDR absencevs. presence	PVDR absence *vs.* presence
BBAAtt	12 (16.2)	2 (16.7)	10 (16.1)	1.04 (0.20–5.48)	1.000	4 (11.1)	8 (21.1)	0.47 (0.13–1.72)	0.246	1 (6.7)	11 (18.6)	0.31 (0.04–2.63)	0.439
BbAATt	4 (5.4)	3 (25.0)	1 (1.6)	20.3 (1.90–217)	0.012	4 (11.1)	0 (–)	–[b]	0.051	0 (–)	4 (6.8)	–[b]	0.576
BbAATT	1 (1.4)	0 (–)	1 (1.6)	–[b]	1.000	0 (–)	1 (2.6)	–[b]	1.000	1 (6.7)	0 (–)	–[b]	0.203
BbAaTT	7 (9.5)	0 (–)	7 (11.3)	–[b]	0.590	5 (13.9)	2 (5.3)	2.90 (0.53–16.0)	0.255	1 (6.7)	6 (10.2)	0.63 (0.07–5.68)	1.000
BbAaTt	24 (32.4)	1 (8.3)	23 (37.1)	0.15 (0.02–1.27)	0.089	13 (36.1)	11 (28.9)	1.39 (0.52–3.68)	0.511	4 (26.7)	20 (33.9)	0.71 (0.20–2.51)	0.761
BbaaTT	2 (2.7)	0 (–)	2 (3.2)	–[b]	1.000	0 (–)	2 (5.3)	–[b]	0.494	0 (–)	2 (3.4)	–[b]	1.000
bbAATT	2 (2.7)	0 (–)	2 (3.2)	–[b]	1.000	0 (–)	2 (5.3)	–[b]	0.494	1 (6.7)	1 (1.7)	4.14 (0.24–70.4)	0.367
bbAaTT	5 (6.8)	3 (25.0)	2 (3.2)	10.0 (1.46–68.3)	0.028	4 (11.1)	1 (2.6)	4.62 (0.49–43.5)	0.194	0 (–)	5 (8.5)	–[b]	0.576
bbaaTT	6 (8.1)	0 (–)	6 (9.7)	–[b]	0.581	1 (2.8)	5 (13.2)	0.19 (0.02–1.70)	0.200	2 (13.3)	4 (6.8)	2.11 (0.35–12.8)	0.595

[a] Over the median value of percentage (%) of cells positive for VDR protein. [b] OR uncountable because one or two of compared groups included zero subject.

Table S3 Genotype and allele characteristics of FokI, BsmI, and TaqI VDR-polymorphisms and BsmI-TaqI haplotypes of 74 melanoma patients and comparisons between groups of 100% VDR-positive cells (*n*=12) *vs.* <100% (*n*=62); over the median (>20%) VDR-positive cells (*n*=36) versus below or equal the median (≤20%) (*n*=38); and absence of VDR-positive cell (*n*=15) versus remaining cases with detected VDR expression (*n*=59)

VDR genotype or combined genotype	All melanoma patients (*n*=74)	100% VDR-positive cells (*n*=12)	<100% VDR-positive cells (*n*=62)	OR (CI) 100% *vs.* <100% VDR-positive	*P*=100% *vs.* <100% VDR-positive	>20%[a] VDR-positive cells (*n*=36)	≤20% VDR-positive cells (*n*=38)	OR (CI)>20% *vs.* ≤ 20% VDR-positive	*P*>20% *vs.* ≤ 20% VDR-positive	VDR absence (0% positive) (*n*=15)	VDR presence (>0% positive) (*n*=59)	OR (CI) VDR absence *vs.* presence	PVDR absence *vs.* presence
FokI genotype													
FF	32 (43.2)	5 (41.7)	27 (43.5)	0.93 (0.26–3.24)	0.904	16 (44.4)	16 (42.1)	1.10 (0.44–2.76)	0.839	7 (46.7)	25 (42.4)	1.19 (0.38–3.71)	0.764
Ff	33 (44.6)	5 (41.7)	28 (45.2)	0.87 (0.25–3.03)	0.824	14 (38.9)	19 (50.0)	0.64 (0.25–1.60)	0.337	6 (40.0)	27 (45.8)	0.79 (0.25–2.50)	0.688
ff	9 (12.2)	2 (16.7)	7 (11.3)	1.57 (0.28–8.69)	0.633	6 (16.7)	3 (7.9)	2.33 (0.54–10.1)	0.302	2 (13.3)	7 (11.9)	1.14 (0.21–6.16)	1.000
F allele	97/148 (65.5)	15/24 (62.5)	82/124 (66.1)	0.85 (0.34–2.11)	0.732	46/72 (63.9)	51/76 (67.1)	0.87 (0.44–1.71)	0.681	20/30 (66.7)	77/118 (65.3)	1.06 (0.46–2.49)	0.884
f allele	51/148 (34.5)	9/24 (37.5)	42/124 (33.9)	1.17 (0.47–2.90	0.732	26/72 (36.1)	25/76 (32.9)	1.15 (0.58–2.27)	0.681	10/30 (33.3)	41/118 (34.7)	0.94 (0.40–2.19)	0.884
BsmI genotype													
BB	23 (31.1)	5 (41.7)	18 (29.0)	1.75 (0.49–6.23)	0.498	9 (25.0)	14 (36.8)	0.57 (0.21–1.56)	0.271	6 (40.0)	17 (28.8)	1.65 (0.51–5.34)	0.533
Bb	38 (51.4)	4 (33.3)	34 (54.8)	0.41 (0.11–1.51)	0.172	22 (61.1)	16 (42.1)	2.16 (0.85–5.47)	0.102	6 (40.0)	32 (54.2)	0.56 (0.18–1.78)	0.325
bb	13 (17.6)	3 (25.0)	10 (16.1)	1.73 (0.40–7.55)	0.432	5 (13.9)	8 (21.1)	0.60 (0.18–2.06)	0.418	3 (20.0)	10 (16.9)	1.22 (0.29–5.15)	0.719

Continued

Continued

VDR genotype or combined genotype	All melanoma patients (*n*=74)	100% VDR-positive cells (*n*=12)	<100% VDR-positive cells (*n*=62)	OR (CI) 100% *vs.* <100% VDR-positive	*P*=100% *vs.* <100% VDR-positive	>20%[a] VDR-positive cells (*n*=36)	≤20% VDR-positive cells (*n*=38)	OR (CI)>20% *vs.* ≤ 20% VDR-positive	*P*>20% *vs.* ≤ 20% VDR-positive	VDR absence (0% positive) (*n*=15)	VDR presence (>0% positive) (*n*=59)	OR (CI) VDR absence *vs.* presence	PVDR absence *vs.* presence
B allele	84/148 (56.8)	14/24 (58.3)	70/124 (56.5)	1.08 (0.44–2.62)	0.865	40/72 (55.6)	44/76 (57.9)	0.91 (0.47–1.74)	0.774	18/30 (60.0)	66/118 (55.9)	1.18 (0.52–2.67)	0.688
b allele	64/148 (43.2)	10/24 (41.7)	54/124 (43.5)	0.93 (0.38–2.24)	0.865	32/72 (44.4)	32/76 (42.1)	1.10 (0.57–2.11)	0.774	12/30 (40.0)	52/118 (44.1)	0.85 (0.37–1.91)	0.688
TaqI genotype													
TT	24 (32.4)	3 (25.0)	21 (33.9)	0.65 (0.16–2.66)	0.740	10 (27.8)	14 (36.8)	0.66 (0.25–1.76)	0.405	6 (40.0)	18 (30.5)	1.52 (0.47–4.90)	0.543
Tt	38 (51.4)	7 (58.3)	31 (50.0)	1.40 (0.40–4.89)	0.597	22 (61.1)	16 (42.1)	2.16 (0.85–5.47)	0.102	8 (53.3)	30 (50.8)	1.10 (0.35–3.44)	0.863
tt	12 (16.2)	2 (16.7)	10 (16.1)	1.04 (0.20–5.48)	1.000	4 (11.1)	8 (21.1)	0.47 (0.13–1.72)	0.246	1 (6.7)	11 (18.6)	0.31 (0.04–2.63)	0.439
T allele	86/148 (58.1)	13/24 (54.2)	73/124 (58.9)	0.83 (0.34–1.99)	0.669	42/72 (58.3)	44/76 (57.9)	1.02 (0.53–1.96)	0.957	20/30 (66.7)	66/118 (55.9)	1.58 (0.68–3.66)	0.287
t allele	62/148 (41.9)	11/24 (45.8)	51/124 (41.1)	1.21 (0.50–2.92)	0.669	30/72 (41.7)	32/76 (42.1)	0.98 (0.51–1.89)	0.957	10/30 (33.3)	52/118 (44.1)	0.63 (0.27–1.47)	0.287
BsmI-TaqI combined genotype													
BBTT	1 (1.4)	0 (–)	1 (1.6)	–[b]	1.000	0 (–)	1 (2.6)	–[b]	1.000	1 (6.7)	0 (–)	–[b]	0.203
BBTt	10 (13.5)	3 (25.0)	7 (11.3)	2.62 (0.57–12.0)	0.351	5 (13.9)	5 (13.2)	1.06 (0.28–4.04)	1.000	4 (26.7)	6 (10.2)	3.21 (0.77–13.3)	0.110
BBtt	12 (16.2)	2 (16.7)	10 (16.1)	1.04 (0.20–5.48)	1.000	4 (11.1)	8 (21.1)	0.47 (0.13–1.72)	0.246	1 (6.7)	11 (18.6)	0.31(0.04–2.63)	0.439
BbTT	10 (13.5)	0 (–)	10 (16.1)	–[b]	0.200	5 (13.9)	5 (13.2)	1.06 (0.28–4.04)	1.000	2 (13.3)	8 (13.6)	0.98 (0.19–5.18)	1.000
BbTt	28 (37.8)	4 (33.3)	24 (38.7)	0.79 (0.21–2.92)	1.000	17 (47.2)	11 (28.9)	2.20 (0.84–5.73)	0.105	4 (26.7)	24 (40.7)	0.53 (0.15–1.86)	0.318
bbTT	13 (17.6)	3 (25.0)	10 (16.1)	2.25 (0.50–10.1)	0.432	5 (13.9)	8 (21.1)	0.60 (0.18–2.06)	0.418	3 (20.0)	10 (16.9)	1.22 (0.29–5.15)	0.719

[a] Over the median value of percentage (%) of cells positive for VDR protein. [b] OR uncountable because one or two of compared groups included zero subject.

Interleukin 1 receptor antagonist gene variable number of tandem repeats polymorphism and cutaneous melanoma

SABINA CAUCI[1], CINZIA BULIGAN[1,2], FRANCESCO ROCCHI[1], ILARIA SALVADOR[1], LUIGI XODO[1] and GIUSEPPE STINCO[1,2]

[1]Department of Medicine, University of Udine; [2]Department of Dermatology, University-Hospital of Udine, I-33100 Udine, Italy

Received April 24, 2019; Accepted July 11, 2019

DOI: 10.3892/ol.2019.10923

Abstract. Immunity and cytokines serve crucial roles in cutaneous melanoma. The present study investigated whether a variable number tandem repeat (VNTR) polymorphism of interleukin-1 receptor antagonist (IL-1RA) gene (*IL-1RN*) located in intron 2 (rs2234663) is associated with cutaneous melanoma. A total of 515 subjects were studied, 133 of which were cutaneous melanoma cases (72 stage I+II non-metastatic melanoma cases and 61 stage III+IV metastatic melanoma cases), and 382 subjects were matching healthy controls from the Friuli-Venezia-Giulia Region located in Northeast Italy, an area with a high melanoma incidence. The *IL-1RN*-VNTR polymorphism was determined by DNA fragment length analysis following PCR amplification. According to the number of 86-bp repeats, five different *IL-1RN* alleles were identified: Allele 1 (4-repeats), allele 2 (2-repeats, short allele), allele 3 (5-repeats), allele 4 (3-repeats) and allele 5 (6-repeats). Alleles with three or more 86-bp repeats, i.e. allele 1, 3, 4 and 5 were collectively denoted as long (L) repeats. The present study revealed that *IL-1RN*-VNTR 1/2 and 2/L genotypes were more frequent among patients with cutaneous melanoma (43.6 and 45.1%, respectively) compared with healthy controls [29.6 and 30.6%, respectively; odds ratio (OR), 1.84; CI, 1.22-2.77; P=0.003; and OR, 1.66; CI, 1.24-2.79; P=0.002, respectively]. Conversely, the *IL-1RN*-VNTR 1/1 genotype was less frequent among melanoma cases (45.9%) compared with healthy controls (57.9%; OR, 0.62; CI, 0.41-0.92; P=0.017). Comparison of metastatic vs. non-metastatic melanoma cases identified no significant differences. The present study first demonstrated that carriage of the 1/1 *IL-1RN*-VNTR genotype was protective, whereas 1/2 and 2/L was a risk factor for patients with cutaneous melanoma vs. healthy controls. The short allele 2 was associated with higher expression levels of IL-1RA, a potent competitive inhibitor of the proinflammatory cytokines IL-1α and IL-1β. VNTR-*IL-1RN* polymorphism may affect susceptibility to melanoma and, thus, it is a potential novel diagnostic biomarker for melanoma. The present study increased the understanding of genetic melanoma susceptibility/carcinogenesis, and may indicate novel strategies in the personalized prevention of cutaneous melanoma.

Correspondence to: Dr Sabina Cauci, Department of Medicine, University of Udine, Piazzale Kolbe 4, I-33100 Udine, Italy
E-mail: sabina.cauci@uniud.it

Abbreviations: IL-1RA, interleukin 1 receptor antagonist; IL-1RN, interleukin 1 receptor antagonist gene; VNTR, variable number of tandem repeats polymorphism; FVG, Friuli Venezia-Giulia; MetM, metastatic melanoma; NMetM, non-metastatic melanoma; BMI, body mass index; TILs, tumor infiltrating lymphocytes

Key words: cutaneous melanoma, skin cancer, polymorphism, interleukin 1 family, interleukin 1 receptor, interleukin 1 receptor antagonist, immunity, innate response

Introduction

Factors that affect cutaneous melanoma need to be addressed and molecular genetic studies appear promising for a precision/personalized medicine approach (1-3). A growing body of data indicates that besides exposure to ultraviolet (UV) radiation, especially intermittent sun exposure (4), sunburns (4,5), and fair skin (4-6), other factors including vitamin D (2,3,7), polychlorinated biphenyls (8), host inflammatory responses (9,10), geographical factors (5,6,11,12), the lifestyle (11-13), and genetic background can also play a role (1-3,14). Melanoma has a rate of 22.0 in males and 18.3 in females per 100,000/year (standardized for European population) in Italy (15), and higher incidence rates (32.5/100,000/year in males; 23.7/100,000/year in females) have been recorded in Friuli Venezia Giulia (FVG) (15), a Region located in Northeast Italy at the border with Austria and Slovenia. The incidence of melanoma in Italy shows an increasing trend (15), Northeast Italy has the highest prevalence of melanoma (16), and the FVG Region has the highest rate of hospitalization for melanoma in Italy (17).

The interleukin 1 alpha (IL-1α) and IL-1 beta (IL-1β) cytokines and the specific receptor antagonist (IL-1RA) are components of the interleukin 1 family (18). IL-1α and IL-1β are involved in several physiological and pathological diseases (18,19), modulating the immune response to pathogenesis and tissue injuries. Moreover, these cytokines have a role in the promotion of cancer-associated inflammation paradoxically both as protective or favoring cancer/metastasis onset (19,20). IL-1RA neutralizes IL-1α and IL-1β action by

binding to the IL-1 receptors (IL-1R type I, IL-1R1 and type II, IL-1R2) without causing any signaling transduction (18). This prevents the IL-1α and IL-1β induced cascade of kinases that activates NF-kB and cyclooxygenase-2 (COX-2) following IL-1 (either α or β) binding to IL-1R1 (18). The IL-1 family of cytokines and receptors is involved in a broad spectrum of immunological and inflammatory responses including activation of lymphocytes (18-22). Specifically, IL-1 cytokines promote increased levels of the main chemokine IL-8 (CXCL8) and consequently activate recruitment of neutrophils (23,24). An *in vitro* human cellular study showed that IL-1RA inhibits CXCL8 release (24). Interestingly, a recent research in myeloid cells found that inflammasomes/IL-1 pathways induce the expression of the programmed death-ligand 1 (PD-L1) on tumor cells, which is an immune checkpoint molecule used as target of melanoma therapy (25,26). Furthermore, blocking IL-1 receptor with IL-1RA or anti-IL-1R1 antibody inhibits tumor growth and metastasis accompanied by decreased accumulation of myeloid cells and expression of the PD-L1 molecule (25).

Currently, inflammation and specific IL-1 targeting as treatment of cancer is an active area of experimental and clinical research (18,20,27). IL-1 mediated inflammation is proposed to contribute to the development and progression of some cancers including melanoma (28). IL-1 appear to act at different levels in tumor initiation and progression, including driving chronic non-resolving inflammation, tumor angiogenesis, activation of the IL-17 pathway, induction of myeloid-derived suppressor cells and macrophage recruitment, invasion and metastasis (20). The effects of IL-1 cytokines are pleiotropic so that any shift of the biological balance between agonistic and antagonistic signals has the potential to cause a disease (19). Notably, the role of IL-1RA in cancer has been studied in different types of tumors showing effects on survival and progression (18-22).

Increasing evidence showed that genetic polymorphisms of IL-1 family members can affect susceptibility to disease. Human IL-1RA gene (*IL-1RN*) is located in chromosome 2, specifically 2q13-2q21 (29,30). Current studies and meta-analyses evaluated the role of a variable number of tandem (VNTR) 86-bp repeats located in intron 2 of the *IL-1RN* gene in relation to various diseases and cancers, particularly gastric cancer (31-33). Specifically, the *IL-1RN* allele 2 (*IL-1RN**2, constituted of two 86-bp repeats also denoted as short allele) has been associated with increased cancer risk in heterozygous subjects (33). Nonetheless, *IL-1RN* VNTR polymorphism roles in cancer still require further study as inconsistent results have been obtained in cancers of different tissue origins (33).

So far, only one German study assessed the role of *IL-1RN* VNTR polymorphism in 97 melanoma patients and 343 controls (34). No significant findings were obtained, the heterozygous 1/2 genotype was found in 28.8% of advanced melanoma patients vs. 39.6% of healthy controls; P=0.06 (34).

Pathways underlying the relationship of *IL-1RN* VNTR and melanoma currently poses an interesting new challenge in melanoma research (1,34) also by considering the potential roles of IL-1RA in modulating PD-1/PD-L1 (25).

Progress in the understanding of melanoma risk factors, genomics, and molecular pathogenesis may drive advances in precision medicine applied to melanoma (1-3,35,36).

In light of these observations, we explored *IL-1RN* VNTR polymorphism and its association with cutaneous malignant melanomas, specifically those with metastatic melanoma (MetM) vs. non-metastatic melanoma (NMetM) and vs. healthy controls.

Materials and methods

Population. Enrolment and clinical visits of all study participants were performed at the Udine University-Hospital Dermatology Clinic. Diagnostic procedures were carried out according to routine protocols. The Udine Institutional Ethical Committee approved the study protocol, which was conducted according to the Declaration of Helsinki. All participants were alive during enrolment in the study and signed a written informed consent.

Using a case-control design, the study consecutively enrolled 133 (age range of 31-87 years) unrelated patients (hospitalized or outpatients) of both sexes with documented cutaneous melanoma diagnosis and 382 (age range of 31-87 years) asymptomatic healthy controls of both sexes, which were matched for age and ancestry with melanoma cases. Inclusion criteria for both melanoma cases and healthy controls were as follows: Caucasian resident in FVG Region, at least two Italian grandparents born in FVG Region (or Austro-Hungarian territory before World War I) as described (2,3). Due to the demonstrated association of *IL-1RN* VNTR with high-grade athleticism, athletes were excluded both from melanoma cases and healthy controls as described (37). Further, exclusion criteria for healthy controls included the following: any kind of lifelong malignant or benign tumor, first-grade relatives with a history of melanoma, and major chronic diseases, such as autoimmune diseases including type 1 diabetes. Among healthy controls 268 subjects were previously studied (37).

Melanoma was diagnosed using immunohistological findings obtained after surgical excision of nevi with clinical and dermoscopic characteristics suggesting the presence of malignancy. Classification of melanoma stages was performed by clinical/histological/radiological findings, as described (38,39), which was our routine in the period of the study whose patient enrollment was completed on December 2017. Inclusion criteria for case-patients comprised only cutaneous not mucosal melanomas. For patients with multiple melanomas, the major melanoma characteristics were accounted for in study analyses according to the histological assessment of major primary tumor (T) grading.

Each participant answered a questionnaire, which was used to collect data on demographic characteristics, medical and family history of melanoma, smoking habits, and history of sunburns as described (2). Phototype was assessed by Fitzpatrick criteria (39). BMI was determined by weight (kg) divided by squared height (m^2); BMI >30 kg/m^2 was considered an indicator of obesity.

Genetic analysis of the VNTR IL-1RN polymorphism. VNTR *IL-1RN* polymorphism was determined, as previously described (40) after extraction of genomic DNA from ethylenediaminetetraacetic-acid-treated venous blood samples (41). The *IL-1RN* intron 2 VNTR polymorphism (rs2234663; also indicated as rs380092) was analyzed using 5'-CTCAGCAAC

ACTCCTAT-3' and 5'-TCCTGGTCTGCAGGTAA-3' as primers (37,40). The PCR products of 412-bp (allele 1 corresponding to 4 repeats of the 86-bp region), 240-bp (allele 2, 2 repeats also defined as short allele), 498-bp (allele 3, 5 repeats), 326-bp (allele 4, 3 repeats), 584-bp (allele 5, 6 repeats) were analyzed by electrophoresis on 10% acrylamide gel stained with ethidium bromide (40). Alleles with three or more 86-bp repeats were denoted as long (L) alleles (37).

Statistical analysis. Continuous variables were expressed as a mean ± standard deviation, and Mann-Whitney *U* test was performed for comparison. Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated for categorical variables, and P values for two-sided Pearson's chi-squared or Fisher's exact test were reported as was appropriate.

P<0.05 was considered to indicate a statistically significant difference and P≤0.10 indicates a tendency to be significant. Statistical software SPSS for Windows (SPSS Inc., Chicago, IL, USA) was used.

Results

Study subjects. All 515 (133 cutaneous melanoma patients and 382 healthy controls) study subjects were Italian Caucasian residents in Northeast Italy. Melanoma cases and healthy controls did not differ for age.

Comparison of VNTR IL-1RN genotypes in cutaneous melanoma patients and healthy controls (as shown in Table I). In all 515 study subjects (data shown in Table SI), the most frequent *IL-1RN* allele was allele 1 *(IL-1RN*1,* frequency was 72.9%), followed by allele 2 *(IL-1RN*2,* frequency was 24.5%) and by allele 3 *(IL-1RN*3*, frequency was 2.5%). The rare heterozygous genotype 1/4 was found in one MetM patient, and 1/5 genotype was found in one NMetM patient, finally, the rare homozygous 3/3 was found in one healthy control.

Homozygous 1/1 genotype was less frequent in melanomas than in healthy control subjects (OR=0.62, P=0.017). Conversely, heterozygous 1/2 genotype was almost twice more frequent in melanomas than in healthy patients (OR=1.84, P=0.003). No differences were observed for 1/3, 2/2, and 2/3 genotypes. The group of all heterozygous genotypes containing the short allele 2 and one of the long alleles 1, 3, 4, 5 (designed in the literature as 2/L genotype) (33,37) had OR=1.66, P=0.002 for melanomas compared with healthy controls. The group including all genotypes containing the short allele 2 (heterozygous 2/L plus homozygous 2/2 genotypes) was more frequent in melanoma patients than in healthy controls (OR=1.53, P=0.036).

As shown in Table I, frequency of allele 1, 2, and 3 did not significantly differ between melanoma and healthy groups, although carriers of allele 1 were slightly less frequent among melanoma patients (70.3%) than healthy controls (74.3%), OR=0.82, P=0.199, whereas carriers of the allele 2 were slightly more frequent among melanoma patients (26.3%) than healthy controls (23.2%), OR=1.18, P=0.300.

Comparisons of IL-1RN VNTR genotypes in MetM and NMetM cutaneous melanoma patients and healthy controls (as shown in Table II). The VNTR *IL-1RN* genotypes were not associated with the metastatic grade of cutaneous melanoma as illustrated in Table II by comparison of MetM with NMetM patients.

By comparison of 61 MetM patients with healthy controls, 1/1 genotype was protective (OR=0.58, P=0.047) and 1/2 was at risk (OR=1.77, P=0.042), the group of 2/L genotypes showed a tendency at risk (OR=1.68, P=0.063).

By comparison of 72 NMetM patients with healthy controls, the 1/2 and 2/L genotypes were at risk (OR=1.90, P=0.013, and OR=2.03, P=0.006, respectively).

The frequency of allele 1, 2, and 3 did not differ between groups as shown in Table II.

Primary demographic and clinical characteristics of melanoma patients and comparison between VNTR IL-1RN 2/L and other remaining genotypes (as shown in Table III). Main demographic and clinical characteristics of the 133 melanoma patients are described in Table III. We examined in detail the differences between heterozygous carriers of the short allele 2 (2/L group, n=60) and the other remaining genotypes (non-2/L group, n=73) in melanoma patients. No differences were noted between groups, however, the frequency of patients with a number of body nevi >50 tended to be higher in 2/L than non-2/L carriers (58.3% vs. 42.5%, OR=1.90, P=0.069).

In a further analysis (data are not shown) by comparison of 2/L carriers with L/L carriers (n=68) among melanoma patients, still non significant differences were noted, however, frequency of patients with a number of body nevi >50 tended to be higher in 2/L than L/L carriers (58.3% vs. 42.6%, OR=1.88, CI=0.93-3.80, P=0.077).

Complete questionnaire data including body nevi number were available for 114 healthy control subjects; no significant differences were noted for a nevi number >50 by comparing 2/L vs. all other remaining genotypes (12/40, 30.0% vs. 25/74, 33.8%, OR=0.84, CI=0.37-1.93, P=0.680), and by comparing 2/L vs. L/L (12/40, 30.0% vs. 23/66, 34.8%, OR=0.80, CI=0.34-1.86, P=0.607).

Discussion

IL-1RA has been implicated in oncogenesis as mice deficient in IL-1RA develop the disease in response to carcinogens (42). IL-1RA by binding to IL-1R1 strongly modulates the action of IL-1 (IL-1α and IL-1β) cytokines, which in turn can induce expression of hundreds of genes, including themselves (via a positive feedback loop) (43).

IL-1RN VNTR polymorphism. *IL-1RN* is considered an important 'hub' gene in melanoma research (1). Previous studies found associations of the short *IL-1RN*2* allele (two 86-bp repeats) with a variety of epithelial-related chronic inflammatory diseases including psoriasis, scleroderma, alopecia areata, lichen sclerosus, systemic lupus erythematosus, and ulcerative colitis (44).

The number of 86-bp repeats in the VNTR *IL-1RN* polymorphism, which is located in intron 2, does not alter the encoded amino acid sequence of IL-1RA, but may be of functional significance as the repeated sequences contain putative binding sites for transcription factors (45,46). A

Table I. Genotype frequencies of *IL-1RN* VNTR in all 515 study subjects and comparison of 133 patients with melanoma with 382 healthy controls.

IL-1RN VNTR genotype/allele	All subjects (n=515) (%)	All patients with melanoma (n=133) (%)	Healthy controls (n=382) (%)	OR (95% CI)	P-value Melanoma vs. Control
1/1	282 (54.8)	61 (45.9)	221 (57.9)	0.62 (0.41-0.92)	0.017
1/2	171 (33.2)	58 (43.6)	113 (29.6)	1.84 (1.22-2.77)	0.003
1/3	18 (3.5)	5 (3.8)	13 (3.4)	1.11 (0.39-3.17)	0.789
2/2	35 (6.8)	5 (3.8)	30 (7.9)	0.46 (0.17-1.21)	0.106
2/3	6 (1.2)	2 (1.5)	4 (1.0)	1.44 (0.26-7.97)	0.651
2/L (1/2 and 2/3)[a]	177 (34.4)	60 (45.1)	117 (30.6)	1.66 (1.24-2.79)	0.002
1/2 and 2/2 and 2/3 and 2/4	212 (41.2)	65 (48.9)	147 (38.5)	1.53 (1.03-2.27)	0.036
1/3 and 2/3 and 3/3[b]	25 (4.9)	7 (5.3)	18 (4.7)	1.12 (0.46-2.75)	0.799
Allele 1	755 (73.3)[c]	187 (70.3)[c]	568 (74.3)	0.82 (0.60-1.11)	0.199
Allele 2	247 (24.0)	70 (26.3)	177 (23.2)	1.18 (0.86-1.63)	0.300
Allele 3	26 (2.5)[d]	7 (2.6)	19 (2.5)[d]	1.06 (0.44-2.55)	0.897

[a]Heterozygous subjects containing the short allele 2 and one long allele (1, 3, 4 and 5) are denoted as 2/L according the literature (33). [b]Homozygous genotype 3/3 was present in only one healthy subject. [c]One metastatic patients had genotype 1/4, and one non-metastatic patient had genotype 1/5. [d]One healthy subject had genotype 3/3. *IL-1RN*, interleukin-1 receptor antagonist gene; OR, odds ratio; VNTR, variable number tandem repeat.

Table II. Genotype frequencies of *IL-1RN* VNTR comparisons of 61 patients with MetM and 72 patients with NMetM and 382 healthy controls.

IL-1RN VNTR genotype/allele	MetM (n=61) (%)	NMetM (n=72) (%)	OR[a] (95% CI)	P-value[a]	OR[b] (95% CI)	P-value[b]	OR[c] (95% CI)	P-value[c]
1/1	27 (44.3)	34 (47.2)	0.89 (0.45-1.76)	0.733	0.58 (0.34-1.00)	0.047	0.65 (0.39-1.08)	0.095[g]
1/2	26 (42.6)	32 (44.4)	0.93 (0.47-1.85)	0.833	1.77 (1.02-3.07)	0.042	1.90 (1.14-3.18)	0.013
1/3	3 (4.9)	2 (2.8)	1.81 (0.29-11.2)	0.660	1.47 (0.41-5.31)	0.472	0.81 (0.18-3.67)	1.000
2/2	4 (6.6)	1 (1.4)	4.98 (0.54-45.8)	0.179	0.82 (0.28-2.42)	1.000	0.16 (0.02-1.23)	0.043
2/3	0 (-)	2 (2.8)	0.97 (0.93-1.01)	0.500	0.99 (0.98-1.00)	1.000	2.70 (0.48-15.0)	0.243
2/L (1/2 and 2/3)[f]	26 (42.6)	34 (47.2)	0.83 (0.42-1.65)	0.595	1.68 (0.97-2.92)	0.063[g]	2.03 (1.21-3.38)	0.006
1/2 and 2/2 and 2/3 and 2/4	30 (49.2)	35 (48.6)	1.02 (0.52-2.02)	0.948	1.55 (0.90-2.66)	0.113	1.51 (0.91-2.51)	0.108
1/3 and 2/3 and 3/3	3 (4.9)	4 (5.5)	0.88 (0.19-4.09)	1.000	1.05 (0.30-3.66)	1.000	1.19 (0.39-3.62)	0.764
Allele 1	84[d] (68.9)	103[e] (71.5)	0.88 (0.52-1.49)	0.634	0.76 (0.50-1.16)	0.201	0.87 (0.58-1.29)	0.480
Allele 2	34 (27.9)	36 (25.0)	1.16 (0.67-2.00)	0.596	1.28 (0.83-1.97)	0.258	1.10 (0.73-1.67)	0.634
Allele 3	3 (2.5)	4 (2.8)	0.88 (0.19-4.02)	1.000	0.99 (0.29-3.39)	1.000	1.12 (0.37-3.34)	0.774

[a]Comparison between MetM and NMetM. [b]Comparison between MetM and healthy controls. [c]Comparison between NMetM and healthy controls. [d]One metastatic patients had genotype 1/4. [e]One non-metastatic patient had genotype 1/5. [f]Heterozygous subjects containing the short allele 2 and one long allele are denoted as 2/L in the literature (33). [g]Significant tendencies. *IL-1RN*, interleukin-1 receptor antagonist gene; MetM, metastatic melanoma; NMetM, non-metastatic melanoma; OR, odds ratio; VNTR, variable number tandem repeat.

general correlation between *IL-1RN*2* allele and the presence of autoinflammatory disease strongly supports a role of VNTR *IL-1RN* polymorphism in the control of the inflammatory response (46).

We observed a general distribution of *IL-1RN* VNTR genotypes (1/1, 54.8%; 1/2, 33.2%; 1/3, 3.5%; 2/2, 6.8%; 2/3, 1.2%; 1/4, 0.2%; 1/5, 0.2% and 3/3, 0.2%) among the 515 Italian study subjects roughly in agreement with other investigations (33,34,40).

Notably, in our study, nearly twofold higher frequency of 1/2 VNTR *IL-1RN* genotype was observed in melanoma patients (43.6%) compared with healthy controls (29.6%), OR=1.84, P=0.003. This OR value became slightly lower (but still statistically significant) by grouping all heterozygous genotypes containing the short 2 allele and one long allele (2/L group, OR=1.66, P=0.002), and all carriers of allele 2 (2/L+2/2) (OR=1.53, P=0.036). We observed that the 1/2 genotype was still at risk by comparing separately the subgroups of metastatic

Table III. Clinical characteristics of 133 patients with melanoma consecutively enrolled, and comparison between the two genetic subgroups of *IL-1RN* VNTR 2/L genotypes (n=60) and all other genotypes (n=73).

Characteristics	All patients with melanoma (n=133)	2/L genotype (n=60)	Non-2/L genotypes (n=73)	OR (CI), 2/L vs. Non-2/L	P-value, 2/L vs. Non-2/L
Age <50 years, n (%)	29 (21.8)	13 (21.7)	16 (21.9)	0.98 (0.43-2.25)	0.972
Age at study enrolment, years, mean ± SD	60.8±12.7	61.1±12.9	60.7±12.7	-	0.788[a]
Age at melanoma diagnosis, years, mean ± SD	54.2±13.7	54.4±13.5	54.0±14.0	-	0.781[a]
Time from melanoma diagnosis, years, mean ± SD	6.7±4.1	6.7±4.4	6.6±3.9	-	0.754[a]
Females, n (%)	58 (43.6)	26 (43.3)	32 (43.8)	0.98 (0.49-1.95)	0.954
Males, n (%)	75 (56.4)	34 (56.7)	41 (56.2)	1.02 (0.51-2.03)	0.954
All grand-parents born in FVG	96 (72.2)	43 (71.7)	53 (72.6)	0.95 (0.45-2.04)	0.905
BMI, kg/m^2, mean ± SD	25.8±3.97	26.1±4.0	25.6±4.0	-	0.643[a]
BMI >30 kg/m^2, n (%)	22 (16.5)	11 (18.3)	11 (15.1)	1.26 (0.51-3.16)	0.614
High school, n (%)	60 (45.1)	27 (45.0)	33 (45.2)	0.99 (0.50-1.97)	0.981
Graduation, n (%)	15 (11.3)	5 (8.3)	10 (13.7)	0.57 (0.18-1.78)	0.330
Present smoker, n (%)	12 (9.0)	4 (6.7)	8 (11.0)	0.58 (0.17-2.03)	0.390
Past smoker, n (%)	53 (39.8)	25 (41.7)	28 (38.4)	1.15 (0.57-2.30)	0.698
Ever smoker, n (%)	65 (48.9)	29 (48.3)	36 (49.3)	0.96 (0.48-1.90)	0.910
≥20 cigarettes ever in all subjects, n (%)	40 (30.1)	17 (28.3)	23 (31.5)	0.86 (0.41-1.81)	0.691
Phototype number	2.4±0.7	2.5±0.7	2.3±0.6	-	0.146[a]
Phototype 1 and 2, n (%)	77 (57.9)	30 (50.0)	47 (64.4)	0.55 (0.28-1.11)	0.095[g]
Nevi > 50, n (%)	66 (49.6)	35 (58.3)	31 (42.5)	1.90 (0.95-3.79)	0.069[g]
Burns over 5, n (%)	71 (53.4)	32 (53.3)	39 (53.4)	1.00 (0.50-1.98)	0.992
NMetM, n (%)	72 (54.1)	34 (56.7)	38 (52.1)	1.20 (0.61-2.39)	0.595
Stage I, n (%)	52 (39.1)	24 (40.0)	28 (38.4)	1.07 (0.53-2.16)	0.847
Stage II, n (%)	19 (14.3)	9 (15.0)	10 (13.7)	1.11 (0.42-2.94)	0.831
Stage III, n (%)	38 (28.6)	19 (31.7)	19 (26.0)	1.32 (0.62-2.80)	0.474
Stage IV, n (%)	24 (18.0)	8 (13.3)	16 (21.9)	0.55 (0.22-1.39)	0.200
Trunk, n (%)	75 (56.4)	36 (60.0)	39 (53.4)	1.31 (0.65-2.61)	0.447
Upper limb, n (%)	9 (6.8)	5 (8.3)	4 (5.5)	1.57 (0.40-6.12)	0.731
Lower limb, n (%)	28 (21.1)	11 (18.3)	17 (23.3)	0.74 (0.32-1.73)	0.486
Hands/feet, n (%)	8 (6.0)	3 (5.0)	5 (6.8)	0.72 (0.16-3.13)	0.729
Head/neck, n (%)	13 (9.8)	5 (8.3)	8 (11.0)	0.74 (0.23-2.39)	0.612
Superficial spreading, n (%)	70 (52.6)	31 (51.7)	39 (53.4)	0.93 (0.47-1.85)	0.840
Nodular, n (%)	45 (33.8)	23 (38.3)	22 (30.1)	1.44 (0.70-2.96)	0.320
Acral lentiginous, n (%)	5 (3.8)	1 (1.7)	4 (5.5)	0.29 (0.03-2.69)	0.378
Lentigo maligna, n (%)	2 (1.5)	0 (-)b	2 (2.7)	0.97 (0.94-1.01)	0.501
Spitzoide, n (%)	5 (3.8)	4 (6.7)	1 (1.4)	5.14 (0.56-47.3)	0.174
Others, n (%)	9 (6.8)	3 (5.0)	6 (8.2)	0.59 (0.14-2.46)	0.512
Breslow thickness, mm, mean ± SD	2.05±1.85	2.17±2.12	1.95±1.61	-	0.871[a]
Clark I, n (%)	2 (1.5)[c]	0 (-)[b,d]	2 (2.8)	0.97 (0.93-1.01)	0.501
Clark II, n (%)	32 (24.4)[c]	15 (25.4)[d]	17 (23.6)[e]	1.10 (0.50-2.45)	0.810
Clark III, n (%)	24 (18.3)[c]	9 (15.3)[d]	15 (20.8)[e]	0.68 (0.27-1.70)	0.411
Clark IV, n (%)	67 (51.1)[c]	33 (55.9)[d]	34 (47.2)[e]	1.42 (0.71-2.83)	0.321
Clark V, n (%)	4 (3.1)[c]	1 (1.7)[d]	3 (4.2)e	0.40 (0.04-3.92)	0.627
Ulceration, n (%)	51 (38.3)	26 (43.3)	25 (34.2)	1.47 (0.73-2.97)	0.284
Mitosis >1, n (%)	83 (63.4)[c]	39 (66.1)[d]	44 (61.1)[e]	1.24 (0.60-2.54)	0.555
Regression, n (%)	20 (15.3)[c]	9 (15.3)[d]	11 (15.3)[e]	1.00 (0.38-2.60)	0.997
Brisk positive TILs, n (%)	38 (29.0)[c]	17 (28.8)[d]	21 (29.2)[e]	0.98 (0.46-2.10)	0.965
Non-brisk TILs, n (%)	47 (35.9)[c]	25 (42.4)[d]	22 (30.6)[e]	1.67 (0.81-3.43)	0.161

Table III. Continued.

Characteristics	All patients with melanoma (n=133)	2/L genotype (n=60)	Non-2/L genotypes (n=73)	OR (CI), 2/L vs. Non-2/L	P-value, 2/L vs. Non-2/L
TILs absence, n (%)	45 (34.4)[c]	17 (28.8)[d]	28 (38.9)[e]	0.64 (0.30-1.33)	0.227
Microsatellitosis, n (%)	5 (3.8)[c]	2 (3.4)[d]	3 (4.2)[e]	0.81 (0.13-5.00)	1.000
Epithelioid variant, n (%)	37 (28.0)[f]	14 (23.3)	23 (31.9)[e]	0.65 (0.30-1.41)	0.273
Fusate variant, n (%)	13 (9.8)[f]	6 (10.0)	7 (9.7)[e]	1.03 (0.33-3.25)	0.957
Small cell variant, n (%)	2 (1.5)[f]	0 (-)[b]	2 (2.8)[e]	0.97 (0.93-1.01)	0.500
More than 1 melanoma, n (%)	20 (15.0)	10 (16.7)	10 (13.7)	1.26 (0.49-3.26)	0.634
Additional non-melanoma skin cancer, n (%)	22 (16.5)	7 (11.7)	15 (20.5)	0.51 (0.19-1.35)	0.170
Additional non-skin cancer, n (%)	29 (21.8)	11 (18.3)	18 (24.7)	0.69 (0.29-1.59)	0.379
Concurrent thyroid disease, n (%)	16 (12.0)	8 (13.3)	8 (11.0)	1.25 (0.44-3.56)	0.675
Melanoma familiarity, n (%)	18 (13.5)	10 (16.7)	8 (11.0)	1.62 (0.60-4.42)	0.338

[a]Two-tailed Mann-Whitney *U*-test. [b]Uncountable because the group contained no subjects. [c]Data were available for 131 patients. [d]Data were available for 59 patients. [e]Data were available for 72 patients. [f]Data were available for 132 patients. [g]Significant tendencies. BMI, body mass index; FVG, Friuli Venezia-Giulia; *IL-1RN*, interleukin-1 receptor antagonist gene; OR, odds ratio; TILs, tumor-infiltrating lymphocytes; VNTR, variable number tandem repeat.

melanomas (OR=1.77, P=0.042) and non-metastatic melanoma (OR=1.90, P=0.013) with healthy controls. Heterozygous 2/L carriers were at increased risk when comparing NMetM vs. healthy controls (OR=2.03, P=0.006), whereas a tendency was observed comparing MetM vs. healthy controls (OR=1.68, P=0.063).

Homozygous VNTR *IL-1RN* 1/1 carriers were at reduced risk comparing melanoma cases with healthy controls (OR=0.62, P=0.017). The 1/1 genotype was protective for the subgroup of metastatic melanomas (OR=0.58, P=0.047), however, only a tendency for protection was observed for non-metastatic melanomas compared to healthy controls (OR=0.65, P=0.095).

By a further analysis, we demonstrated that among melanoma patients the 1/2 and 2/L genotypes frequencies did not differ between MetM and NMetM patients. The 2/2 genotype was more frequent in MetM (6.6%) than in NMetM (1.4%) patients, however, such difference was not statistically significant (OR=4.98, P=0.179).

Our study shows in detail that among melanoma patients, the 2/L genotypes did not differ from the remaining genotypes for demographic characteristics, gender, known risk factors, staging, location, and deepening of melanoma. However, we observed that among melanomas 58.3% of 2/L carries had more than 50 body nevi vs. 42.5% of non-2/L carriers OR=1.90, P=0.069. This tendency result should be confirmed in enlarged studies. A possible association of 2/L genotype with an increased number of body nevi could explain the higher risk of 2/L carriers for melanoma because an elevated body nevi number is a recognized risk factor for melanoma.

This study is the first investigation of VNTR *IL-1RN* polymorphism in Italian melanoma patients, and the second one on this polymorphism and melanoma after the study of Broer and colleagues (34). Broer and colleagues performed a study in aggressive melanomas having stage III or higher (in other words all metastatic melanomas) and 343 healthy controls finding a frequency of allele *IL-1RN**1 of 73.2% in melanomas and of 71.7% in healthy controls, a frequency of allele *IL-1RN**2 of 23.7% in melanomas and of 28.3% in healthy controls, whereas the rare allele 3 was found in 2 melanoma patients, and allele 4 was found in 4 melanoma patients. Those data are roughly in line with our allele frequency data. However, the German study (34) did not observe statistically significant differences between aggressive melanomas and healthy controls for VNTR *IL-1RN* genotypes; indeed the frequency of 1/2 genotype tended to be even lower in aggressive melanomas than controls (28.8% vs. 39.6%, P=0.06). At variance, in our Italian melanoma group, the frequency of 1/2 genotype was 1.5-fold higher (43.6%) than in German patients. Such a difference could derive by different ethnic background (33) and/or by selection of melanoma cases. Moreover, the high rate of 1/2 genotype in the German healthy controls could be due to different inclusion/exclusion criteria in respect to our study, for example, in our study we excluded high-grade athletes because they have an increased frequency of VNTR 1/2 genotype (37).

The 2/L genotype data of our study are consistent with studies relative to other cancers (33,47). The disproportionate levels of IL-1RA could activate balancing elevation of other factors in the complex network of IL-1 family cytokines and receptors that lead to fine tuning of immune response (19,48,49).

Despite a large number of studies, the association of VNTR *IL-1RN* with cancer still shows some inconsistencies (33). The association of the VNTR *IL-1RN* polymorphism with cancer was examined by Zhang and colleagues (33) who performed a meta-analysis including 14,854 cases and 19,337 controls from 71 published case-control studies. Genotypic analysis showed significant associations in gastric cancer (2/L vs. L/L, OR=1.22, CI=1.05-1.41). However, in breast cancer, 2/L vs. L/L was protective (OR=0.74, CI=0.58-0.93), whereas in hepatocellular, cervical and lung cancer data were not significant. Moreover, such positive association with cancer

was stronger in Asian than in Caucasian population (33). The inconsistency is likely caused in part by the differences in the subject ethnicity, sample sizes, disease stages, and cancer types for studies. It is apparent that further studies with large homogeneous patient populations will be needed to validate the association between VNTR *IL-1RN* gene polymorphism and human cancer.

Allele 2 of VNTR IL-1RA polymorphism (*IL-1RN**2) is considered to have modulatory effects on inflammatory response, however, evidence on final effects associated to *IL-1RN**2 are contradictory (48,50,51). Evaluation of comprehensive effects of *IL-1RN**2 is complicated by the concurrent modulation of the anti-inflammatory IL-1RA and the pro-inflammatory IL-1 cytokine levels (48,52). The presence of the *IL-1RN**2 has been associated with enhanced IL-1β production *in vitro* (48), and increased inflammatory response (31,51). Indeed, IL-1RA plasma levels are coordinately regulated by both IL-1RA and IL-1beta genes (53) indicating a cross-regulation between the receptor antagonist and IL-1 cytokine expression (48,49,54). Tissue-specific effects are also possible (33).

According to some studies the carriers of *IL-1RN**2 have higher IL-1RA levels (53,55,56), and consequently the 2 allele effects could down-regulate IL-1 mediated pro-inflammatory signaling pathways by the IL-1RA blocking of IL-1R1 (37,57,58). A recent study (59) showed that individuals with genotype 2/2 VNTR *IL-1RN* exhibited higher IL-1RA expression compared to 1/2 and 1/1 genotypes. The same study indicated that *IL-1RN**2 might be a risk factor for progressive vitiligo (59).

On the basis of such evidence, in *IL-1RN**2 carrier the increased IL-1RA expression could provoke a reduced anti-tumor immune capacity and could favor the onset of melanoma. On the other hand, a study (28) showed that blocking of IL-1R1 by treatment with IL-1R1 neutralizing antibody or IL-1 pathway-specific siRNAs led to growth arrest in IL-1-positive melanoma cells. Furthermore, blocking the IL-1 pathway increased autophagy in IL-1-positive melanoma cells indicating that the endogenous IL-1 system is functional in most human melanoma and interrupting its signaling inhibits the growth of IL-1-positive melanoma cells (28).

It appears plausible that a low IL-1-related immune response (due to moderately elevated IL-1RA as in 2/L genotypes) increases the risk to develop melanoma, but, at the same time, does not increase or even could reduce the risk to develop an aggressive tumor. Such a hypothesis would fit with our present findings showing that 2/L genotype increases the risk to develop melanoma, but is not more frequent in metastatic than non-metastatic melanomas. Furthermore, such hypothesis would also fit with Broer *et al* (34) data showing a tendency of lower frequency of 2/L in aggressive melanomas than healthy controls.

IL-1R1 receptor activation by IL-1α and/or IL-1β induces an array of factors including IL-1, IL-6 (CXCL6), IL-8 (CXCL8), interferon (IFN) α, β, and γ, defensins, matrix metalloproteinases (MMPs), C-reactive protein, etc (18,20,60). Thus, the IL-1RA inhibition of IL-1R1 affects several immune factors. It is worth to note that IL-1RA can compete with IL-1 cytokines also for the IL-1R2, which acts as a decoy receptor. Of note, anakinra (a recombinant form of IL-1RA used as an anti-inflammatory drug in certain diseases) or genetic inactivation of the IL-1β-IL-1R1 system can lead to less melanoma growth in mice (18,28).

A recent study (61) showed that in MyD88-/- mice and in C57BL/6 mice treated with anakinra the relapse rates of mice subcutaneous B16 melanoma tumor growth significantly increase. This study suggested that IL-1, via its action on neutrophils, promotes the anti-cancer efficacy of ingenol mebutate (a drug approved for the topical treatment of actinic keratoses that could ultimately also find utility in treating skin cancers), with ingenol mebutate treatment causing both IL-1β induction and IL-1α release from keratinocytes (61).

Future research should focus on complex gene interactions and biological pathways related to the IL-1 family of cytokines and receptors and melanoma. Improved comprehension of biomolecular immune pathways will support further progress in melanoma management (1-3,33,36).

Study limitations and strengths. A strong point of our study is the highly defined ethnic background of subjects. This variable is important in genetic studies. Specifically, evidence on ethical and geographical variability of genetic polymorphisms in IL-1 family genes is growing (23,33). Moreover, variability in racial distribution and genetic melanoma susceptibility among (and across) different countries suggests that melanoma studies should be performed in restricted and well-characterized ethnic groups (6,11). On the other hand, our results cannot be generalized to populations with different genetic backgrounds. A strength of this study is detailed clinical data of melanoma patients. Limitations of our study include a limited sample size in subgroups of patients and high CIs for some categorical variables. These limitations could have influenced the non-significant results showed in Table II. Thus, future large-scale studies are necessary to better assess the role of such variables.

More personalized approach to cancer is a challenge of current research (62,63). Personalized boosting of anti-tumor immunity in advanced melanomas is a new promising trend in the treatment of malignant melanoma. Immunotherapy is successful for some patients without relapse or progression, but many patients undergoing therapy have progressive disease (9,64,65). Thus, a better understanding of immune factors' modulation contributing to the development of melanoma may increase the likelihood of future improvements in patient management and melanoma prevention by tailored immune modulation modality. IL-1 cytokines and receptors including IL-1RA act in a complex balance, which can both host protect and harm (19). Our data highlighted that in terms of *IL-1RN* gene alteration by VNTR in intron 2, IL-1RA homeostasis plays roles in cutaneous melanoma. *IL-1RN* 2/L (mainly constituted by 1/2 genotype) genotype was found associated to susceptibility to cutaneous melanoma, whereas 1/1 was protective. Thus, our findings support an IL-1 family of receptors contribution to the development of malignant melanoma, suggesting the value of genetic screening as an adjuvant of immune strategies for cancer prevention. Future studies should further explore *IL-1RN* polymorphisms for their inclusion in risk models for individualized prevention/susceptibility/prognosis in the practice of precision medicine applied in cutaneous melanoma.

We first suggest that the heterozygous subjects having the short allele *IL-1RN**2 are more prone to cutaneous malignant melanoma showing that (innate) immune mechanisms play a role in the susceptibility/pathogenesis of this cancer. Interrelationships of *IL-1RN* 86-bp VNTR with other polymorphisms including those of IL-1β gene (IL-1B) are of interest for future research (40,53). *IL-1RN* may be a candidate gene for melanoma pathogenesis or may possibly be a linked marker to other, as yet undefined, genes. However, it is tempting to speculate that determination of IL-1 family polymorphisms could be used in the future for a personalized preventive treatment of healthy subjects at high risk to develop melanoma and/or for prognostic evaluation. Notably, IL-1RA seems to have roles in PD-L1 regulation, which is a main target of immune therapy for advanced melanoma (25). Interestingly, in our study *IL-1RN* 2/L genotype appears to act as a risk factor for melanoma susceptibility independently by conventional risk factors for melanoma, with the possible exception of the elevated presence of nevi. Further investigations are necessary to extend our findings to also examine different ethnic groups (66) and to identify biological pathways related to IL-1RA, which influence skin diseases (67). It is likely that cancer treatment and management will be supplemented in the future by extensive systematic assessment of DNA pathways (1,35,36).

Acknowledgements

The authors would like to thank Professor Silvio Brusaferro (Department of Medicine, Udine University, Udine, Italy) for his help in enrolment of healthy subjects, and technicians Ms. Patrizia Nacci and Mr. Luca Bazzichetto (Department of Medicine, Udine University, Udine, Italy) for their help with experimental analyses. Lastly, the authors would like to express their gratitude to Professor Carlo Pucillo (Department of Medicine, Udine University, Udine, Italy) for critical reading of the manuscript.

Funding

The present study was supported by University of Udine grants between 2015 and 2018.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request

Authors' contributions

SC, CB, LX and GS were responsible for research creation and design, and provided study material or patients. SC, CB, FR, IS, LX and GS collected, assembled, analysed and interpreted the data, and drafted and finalized the manuscript. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

All experiments were approved by the Ethical Committees of Udine Institutional Ethical Committee (Udine, Italy).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Wang LX, Li Y and Chen GZ: Network-based co-expression analysis for exploring the potential diagnostic biomarkers of metastatic melanoma. PLoS One 13: e0190447, 2018.
2. Cauci S, Maione V, Buligan C, Linussio M, Serraino D and Stinco G: BsmI (rs1544410) and FokI (rs2228570) vitamin D receptor polymorphisms, smoking, and body mass index as risk factors of cutaneous malignant melanoma in northeast Italy. Cancer Biol Med 14: 302-318, 2017.
3. La Marra F, Stinco G, Buligan C, Chiriacò G, Serraino D, Di Loreto C and Cauci S: Immunohistochemical evaluation of vitamin D receptor (VDR) expression in cutaneous melanoma tissues and four VDR gene polymorphisms. Cancer Biol Med 14: 162-175, 2017.
4. Gandini S, Montella M, Ayala F, Benedetto L, Rossi CR, Vecchiato A, Corradin MT, DE Giorgi V, Queirolo P, Zannetti G, *et al*: Sun exposure and melanoma prognostic factors. Oncol Lett 11: 2706-2714, 2016.
5. Bonin S, Albano A, di Meo N, Gatti A, Stinco G, Zanconati F and Trevisan G: Cutaneous melanoma frequencies and seasonal trend in 20 years of observation of a population characterised by excessive sun exposure. Radiol Oncol 49: 379-385, 2015.
6. Ferlay J, Colombet M, Soerjomataram I, Dyba T, Randi G, Bettio M, Gavin A, Visser O and Bray F: Cancer incidence and mortality patterns in Europe: Estimates for 40 countries and 25 major cancers in 2018. Eur J Cancer 103: 356-387, 2018.
7. Newton-Bishop JA, Davies JR, Latheef F, Randerson-Moor J, Chan M, Gascoyne J, Waseem S, Haynes S, O'Donovan C and Bishop DT: 25-Hydroxyvitamin D2/D3 levels and factors associated with systemic inflammation and melanoma survival in the Leeds melanoma cohort. Int J Cancer 136: 2890-2899, 2015.
8. IARC: International Agency for Research on Cancer. http://monographs.iarc.fr/ENG/Classification/. Accessed April 17, 2019.
9. Danielli R, Cisternino F, Giannarelli D, Calabrò L, Camerini R, Savelli V, Bova G, Dragonetti R, Di Giacomo AM, Altomonte M and Maio M: Long-term follow up of metastatic melanoma patients treated with thymosin alpha-1: Investigating immune checkpoints synergy. Expert Opin Biol Ther 18 (Suppl 1): S77-S83, 2018.
10. Isvoranu G, Surcel M, Huică RI, Munteanu AN, Pîrvu IR, Ciotaru D, Constantin C, Bratu O, Neagu M and Ursaciuc C: Natural killer cell monitoring in cutaneous melanoma-new dynamic biomarker. Oncol Lett 17: 4197-4206, 2019.
11. Cecconi L, Busolin A, Barbone F, Serraino D, Chiarugi A, Biggeri A and Catelan D: Spatial analysis of incidence of cutaneous melanoma in the Friuli Venezia Giulia region in the period 1995-2005. Geospat Health 11: 422, 2016.
12. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA and Jemal A: Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin 68: 394-424, 2018.
13. Jiang AJ, Rambhatla PV and Eide MJ: Socioeconomic and lifestyle factors and melanoma: A systematic review. Br J Dermatol 172: 885-915, 2015.
14. EUCAN (2012): Malignant melanoma of the skin. ecoi-arcfr/eucan/Canceraspx?Cancer=20. 2012: Accessed April 17, 2019.
15. The Italian Association of Cancer Registries, AIRTUM Working Group. I numeri del cancro in Italia-2018. 2018 http://registri-tumori.it/itacan. Accessed April 17, 2019.
16. AIRTUM (Associazione Italiana Registri Tumori) Working Group. Italian cancer figures-Report 2014. Prevalence and cure of cancer in Italy. Epidemiol Prev 38 (Suppl 1): S1-S144, 2014.
17. Piscitelli P, Neglia C, Falco A, Rivezzi M, Agnello N, Argentiero A, Chitano G, Distante C, Della Rosa G, Vinci G, *et al*: Melanoma in the Italian population and regional environmental influences: A national retrospective survey on 2001-2008 hospitalization records. Int J Environ Res Public Health 12: 9102-9118, 2015.

18. Dinarello CA: Overview of the IL 1 family in innate inflammation and acquired immunity. Immunol Rev 281: 8-27, 2018.
19. Khazim K, Azulay EE, Kristal B and Cohen I: Interleukin 1 gene and susceptibility to disease. Immunol Rev 281: 40-56, 2018.
20. Mantovani A, Barajon I and Garlanda C: IL-1 and IL-1 regulatory pathways in cancer progression and therapy. Immunol Rev 281: 57-61, 2018.
21. Malik A and Kanneganti TD: Function and regulation of IL-1α in inflammatory diseases and cancer. Immunol Rev 281: 124-137, 2018.
22. Garlanda C, Dinarello CA and Mantovani A: The interleukin-1 family: Back to the future. Immunity 39: 1003-1018, 2013.
23. Cauci S, Guaschino S, De Aloysio D, Driussi S, De Santo D, Penacchioni P and Quadrifoglio F: Interrelationships of interleukin-8 with interleukin-1beta and neutrophils in vaginal fluid of healthy and bacterial vaginosis positive women. Mol Hum Reprod 9: 53-58, 2003.
24. Marriott HM, Gascoyne KA, Gowda R, Geary I, Nicklin MJ, Iannelli F, Pozzi G, Mitchell TJ, Whyte MK, Sabroe I and Dockrell DH: Interleukin-1β regulates CXCL8 release and influences disease outcome in response to Streptococcus pneumoniae, defining intercellular cooperation between pulmonary epithelial cells and macrophages. Infect Immun 80: 1140-1149, 2012.
25. Guo B, Zhang J and Fu S: Inflammasomes/IL-1 pathways in myeloid cells modulate PD-1/PD-L1 checkpoint molecules. J Immunol 198 (Suppl 1): S130.24, 2017.
26. Rotte A, Bhandaru M, Zhou Y and McElwee KJ: Immunotherapy of melanoma: Present options and future promises. Cancer Metastasis Rev 34: 115-128, 2015.
27. Coussens LM, Zitvogel L and Palucka AK: Neutralizing tumor-promoting chronic inflammation: A magic bullet? Science 339: 286-291, 2013.
28. Qin Y, Ekmekcioglu S, Liu P, Duncan LM, Lizée G, Poindexter N and Grimm EA: Constitutive aberrant endogenous interleukin-1 facilitates inflammation and growth in human melanoma. Mol Cancer Res 9: 1537-1550, 2011.
29. Webb AC, Collins KL, Auron PE, Eddy RL, Nakai H, Byers MG, Haley LL, Henry WM and Shows TB: Interleukin-1 gene (IL1) assigned to long arm of human chromosome 2. Lymphokine Res 5: 77-85, 1986.
30. Nicklin MJ, Weith A and Duff GW: A physical map of the region encompassing the human interleukin-1 alpha, interleukin-1 beta, and interleukin-1 receptor antagonist genes. Genomics 19: 382-384, 1994.
31. El-Omar EM, Carrington M, Chow WH, McColl KE, Bream JH, Young HA, Herrera J, Lissowska J, Yuan CC, Rothman N, *et al*: Interleukin-1 polymorphisms associated with increased risk of gastric cancer. Nature 404: 398-402, 2000.
32. Perri F, Piepoli A, Bonvicini C, Gentile A, Quitadamo M, Di Candia M, Cotugno R, Cattaneo F, Zagari MR, Ricciardiello L, *et al*: Cytokine gene polymorphisms in gastric cancer patients from two Italian areas at high and low cancer prevalence. Cytokine 30: 293-302, 2005.
33. Zhang Y, Liu C, Peng H, Zhang J and Feng Q: IL1 receptor antagonist gene IL1-RN variable number of tandem repeats polymorphism and cancer risk: A literature review and meta-analysis. PLoS One 7: e46017, 2012.
34. Broer PN, Aung T, Heidekrueger PI, Prantl L and Narayan D: Divisive influence of interleukin-1 receptor antagonist polymorphisms in melanoma patients. Clin Hemorheol Microcirc 67: 319-326, 2017.
35. Sokolenko AP and Imyanitov EN: Molecular tests for the choice of cancer therapy. Curr Pharm Des 23: 4794-4806, 2017.
36. Gu F, Chen TH, Pfeiffer RM, Fargnoli MC, Calista D, Ghiorzo P, Peris K, Puig S, Menin C, De Nicolo A, *et al*: Combining common genetic variants and non-genetic risk factors to predict risk of cutaneous melanoma. Hum Mol Genet 27: 4145-4156, 2018.
37. Cauci S, Di Santolo M, Ryckman KK, Williams SM and Banfi G: Variable number of tandem repeat polymorphisms of the interleukin-1 receptor antagonist gene IL-1RN: A novel association with the athlete status. BMC Med Genet 11: 29, 2010.
38. Balch CM, Gershenwald JE, Soong SJ, Thompson JF, Atkins MB, Byrd DR, Buzaid AC, Cochran AJ, Coit DG, Ding S, *et al*: Final version of 2009 AJCC melanoma staging and classification. J Clin Oncol 27: 6199-6206, 2009.
39. Fitzpatrick TB: The validity and practicality of sun-reactive skin types I through VI. Arch Dermatol 124: 869-871, 1988.
40. Cauci S, Di Santolo M, Casabellata G, Ryckman K, Williams SM and Guaschino S: Association of interleukin-1beta and interleukin-1 receptor antagonist polymorphisms with bacterial vaginosis in non-pregnant Italian women. Mol Hum Reprod 13: 243-250, 2007.
41. Colombini A, Brayda-Bruno M, Lombardi G, Croiset SJ, Ceriani C, Buligan C, Barbina M, Banfi G and Cauci S: BsmI, ApaI and TaqI polymorphisms in the vitamin D receptor gene (VDR) and association with lumbar spine pathologies: An Italian case-control study. PLoS One 11: e0155004, 2016.
42. Zitvogel L, Kepp O, Galluzzi L and Kroemer G: Inflammasomes in carcinogenesis and anticancer immune responses. Nat Immunol 13: 343-351, 2012.
43. Dinarello CA: Introduction to the interleukin-1 family of cytokines and receptors: Drivers of innate inflammation and acquired immunity. Immunol Rev 281: 5-7, 2018.
44. Tarlow JK, Cork MJ, Clay FE, Schmitt-Egenolf M, Crane AM, Stierle C, Boehncke WH, Eiermann TH, Blakemore AI, Bleehen SS, *et al*: Association between interleukin-1 receptor antagonist (IL-1ra) gene polymorphism and early and late-onset psoriasis. Br J Dermatol 136: 147-148, 1997.
45. Tarlow JK, Blakemore AI, Lennard A, Solari R, Hughes HN, Steinkasserer A and Duff GW: Polymorphism in human IL-1 receptor antagonist gene intron 2 is caused by variable numbers of an 86-bp tandem repeat. Hum Genet 91: 403-404, 1993.
46. Clay FE, Tarlow JK, Cork MJ, Cox A, Nicklin MJ and Duff GW: Novel interleukin-1 receptor antagonist exon polymorphisms and their use in allele-specific mRNA assessment. Hum Genet 97: 723-726, 1996.
47. Hishida A, Okugawa Y, Morimoto Y, Shirai Y, Okamoto K, Momokita S, Ogawa A, Tanaka K, Nishikawa R, Toiyama Y, *et al*: Genetic influence of cytokine polymorphisms on the clinical outcome of Japanese gastrointestinal cancer patients in palliative care. Oncol Lett 17: 623-629, 2019.
48. Santtila S, Savinainen K and Hurme M: Presence of the IL-1RA allele 2 (IL1RN*2) is associated with enhanced IL-1beta production in vitro. Scand J Immunol 47: 195-198, 1998.
49. Danis VA, Millington M, Hyland VJ and Grennan D: Cytokine production by normal human monocytes: Inter-subject variation and relationship to an IL-1 receptor antagonist (IL-1Ra) gene polymorphism. Clin Exp Immunol 99: 303-310, 1995.
50. Rafiq S, Stevens K, Hurst AJ, Murray A, Henley W, Weedon MN, Bandinelli S, Corsi AM, Guralnik JM, Ferrucci L, *et al*: Common genetic variation in the gene encoding interleukin-1-receptor antagonist (IL-1RA) is associated with altered circulating IL-1RA levels. Genes Immun 8: 344-351, 2007.
51. Reiner AP, Wurfel MM, Lange LA, Carlson CS, Nord AS, Carty CL, Rieder MJ, Desmarais C, Jenny NS, Iribarren C, *et al*: Polymorphisms of the IL1-receptor antagonist gene (IL1RN) are associated with multiple markers of systemic inflammation. Arterioscler Thromb Vasc Biol 28: 1407-1412, 2008.
52. Dewberry R, Holden H, Crossman D and Francis S: Interleukin-1 receptor antagonist expression in human endothelial cells and atherosclerosis. Arterioscler Thromb Vasc Biol 20: 2394-2400, 2000.
53. Hurme M and Santtila S: IL-1 receptor antagonist (IL-1Ra) plasma levels are co-ordinately regulated by both IL-1Ra and IL-1beta genes. Eur J Immunol 28: 2598-2602, 1998.
54. Buchs N, di Giovine FS, Silvestri T, Vannier E, Duff GW and Miossec P: IL-1B and IL-1Ra gene polymorphisms and disease severity in rheumatoid arthritis: Interaction with their plasma levels. Genes Immun 2: 222-228, 2001.
55. Bioque G, Crusius JB, Koutroubakis I, Bouma G, Kostense PJ, Meuwissen SG and Peña AS: Allelic polymorphism in IL-1 beta and IL-1 receptor antagonist (IL-1Ra) genes in inflammatory bowel disease. Clin Exp Immunol 102: 379-383, 1995.
56. Strandberg L, Lorentzon M, Hellqvist A, Nilsson S, Wallenius V, Ohlsson C and Jansson JO: Interleukin-1 system gene polymorphisms are associated with fat mass in young men. J Clin Endocrinol Metab 91: 2749-2754, 2006.
57. Ramírez-Pérez S, De la Cruz-Mosso U, Hernández-Bello J, Martínez-Bonilla GE, Ramírez-Dueñas MG, Pereira-Suárez AL, Parra Rojas I, Martínez-López E, Macías-Barragán J and Muñoz-Valle JF: High expression of interleukin-1 receptor antagonist in rheumatoid arthritis: Association with IL1RN*2/2 genotype. Autoimmunity 50: 468-475, 2017.

58. Živković M, Kolić I, Jesić S, Jotić A and Stanković A: The allele 2 of the VNTR polymorphism in the gene that encodes a natural inhibitor of IL-1β, IL-1RA is favorably associated with chronic otitis media. Clin Exp Otorhinolaryngol 11: 118-123, 2018.
59. Singh M, Mansuri MS, Jadeja SD, Marfatia YS and Begum R: Association of interleukin 1 receptor antagonist intron 2 variable number of tandem repeats polymorphism with vitiligo susceptibility in Gujarat population. Indian J Dermatol Venereol Leprol 84: 285-291, 2018.
60. Acuner Ozbabacan SE, Gursoy A, Nussinov R and Keskin O: The structural pathway of interleukin 1 (IL-1) initiated signaling reveals mechanisms of oncogenic mutations and SNPs in inflammation and cancer. PLoS Comput Biol 10: e1003470, 2014.
61. Le TT, Skak K, Schroder K, Schroder WA, Boyle GM, Pierce CJ and Suhrbier A: IL-1 contributes to the anti-cancer efficacy of ingenol mebutate. PLoS One 11: e0153975, 2016.
62. Amin MB, Greene FL, Edge SB, Compton CC, Gershenwald JE, Brookland RK, Meyer L, Gress DM, Byrd DR and Winchester DP: The Eighth Edition AJCC Cancer Staging Manual: Continuing to build a bridge from a population-based to a more 'personalized' approach to cancer staging. CA Cancer J Clin 67: 93-99, 2017.
63. Kattan MW, Hess KR, Amin MB, Lu Y, Moons KG, Gershenwald JE, Gimotty PA, Guinney JH, Halabi S, Lazar AJ, *et al*: American Joint Committee on Cancer acceptance criteria for inclusion of risk models for individualized prognosis in the practice of precision medicine. CA Cancer J Clin 66: 370-374, 2016.
64. Keller HR, Zhang X, Li L, Schaider H and Wells JW: Overcoming resistance to targeted therapy with immunotherapy and combination therapy for metastatic melanoma. Oncotarget 8: 75675-75686, 2017.
65. Yu Y and Cui J: Present and future of cancer immunotherapy: A tumor microenvironmental perspective. Oncol Lett 16: 4105-4113, 2018.
66. Abbasian MH, Abbasi B, Ansarinejad N, Motevalizadeh Ardekani A, Samizadeh E, Gohari Moghaddam K and Hashemi MR: Association of interleukin-1 gene polymorphism with risk of gastric and colorectal cancers in an Iranian population. Iran J Immunol 15: 321-328, 2018.
67. Qiao J, Jia QN and Jin HZ: Lack of association of the IL-1RN and IL-10 polymorphisms with risk of psoriasis: A meta-analysis. Mol Genet Genomic Med 7: e00512, 2019.