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RESEARCH ARTICLE

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Molecular investigation of some DNA viruses in mucosal melanoma: Case–control study

Rosaria Arvia[1] | Michele Tanturli[2] | Filippo Ugolini[3] | Margherita Vannucchi[4] | Daniela Massi[3] | Krystyna Zakrzewska[1]

[1]Department of Experimental and Clinical Medicine, University of Florence, Florence, Italy

[2]Department of Experimental and Clinical Biomedical Sciences "Mario Serio", University of Florence, Florence, Italy

[3]Department of Health Sciences, University of Florence, Florence, Italy

[4]Department of Medical Biotechnology, Section of Pathology, University of Siena, Siena, Italy

Correspondence
Rosaria Arvia, Department of Clinical and Experimental Medicine, University of Florence, Florence, Italy, Viale Morgagni 48, 50134 Florence, Italy.
Email: rosaria.arvia@unifi.it

Abstract

The association between viral infections and both cutaneous and mucosal melanoma (MM) has not been fully investigated. Here, we assessed the prevalence of the DNA of a broad range of viruses in 31 MMs and 15 biopsies of healthy mucosa (HM) using molecular methods. The parvoviruses CuV and B19V, herpesviruses HSV1, HSV2, EBV, HHV6, and HHV8, polyomavirus MCPyV, and α-HPVs were not detected, or rarely found, in MMs, and in HM, of the digestive, respiratory, and female genital tract. The overall prevalence of β-HPV in MMs was not significantly higher compared to that in HM (70.9% and 53.3% respectively; $p = 0.514$). However, the number of MMs positive for β-HPV types belonging to Species 3 and 5 and for some viral types belonging to Species 1, 2, 3, and 5 were significantly higher compared with HM ($p < 0.05$). Moreover, compared to HM, the MM samples contained a significantly higher number of β-HPV types, mainly belonging to Species 1, 3, and 5 ($p < 0.05$). Our data, although suggesting a role for certain β-HPV types in MM oncogenesis, require additional investigation in larger populations to support this hypothesis.

KEYWORDS

β-HPV, mucosal melanomas, oncogenic viruses

1 | INTRODUCTION

Mucosal melanoma (MM) is a rare tumor, accounting for only 0.03% of all cancers and for 1.3% of all melanomas.[1] However, MM is clinically relevant because it is more aggressive and has a worse prognosis than the more common cutaneous melanoma (CM).[1]

The main risk factors for CM include ultraviolet (UV) radiation, fair skin, a high number of acquired melanocytic nevi, and family history.[2] Conversely, exposure to UV radiation does not appear to be a risk factor for MM. To date, the etiology of MMs is still unknown and genetic and/or environmental risk factors have not been clearly identified.[3] Although a direct correlation between cigarette smoking and MM could not be demonstrated, it is known that 66% of patients with melanoma of the nasopharyngeal tract report a history of smoking.[4,5] Moreover, vulvovaginal MM is associated with chronic inflammation, local trauma, use of irritant agents, and family history of CM,[3] while anorectal MM seems to be more common in patients with HIV infection.

It is known that some viral infections are associated with a variety of human cancers. However, the role of viruses, as a risk factor for melanoma has not been fully investigated.

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TABLE 1 Characteristics of the tissue samples analyzed in the study.

Case	Sample	Sex	Age	Anatomical site
1	MM	F	57	Digestive tract
3	MM	F	75	Digestive tract
5	MM	F	80	Digestive tract
6	MM	M	67	Digestive tract
8	MM	M	75	Digestive tract
10	MM	F	77	Digestive tract
11	MM	M	82	Digestive tract
12	MM	M	87	Digestive tract
13	MM	M	73	Digestive tract
14	MM	F	73	Digestive tract
15	MM	F	NA	Digestive tract
16	MM	F	63	Genital tract
17	MM	F	98	Genital tract
20	MM	F	69	Genital tract
22	MM	F	69	Genital tract
24	MM	F	84	Genital tract
25	MM	F	74	Genital tract
27	MM	F	86	Genital tract
31	MM	F	45	Genital tract
32	MM	F	69	Respiratory tract
33	MM	M	77	Respiratory tract
34	MM	F	72	Respiratory tract
35	MM	F	97	Respiratory tract
36	MM	F	72	Respiratory tract
37	MM	M	80	Respiratory tract
1 C	MM	F	85	Genital tract
2 C	MM	M	64	Digestive tract
3 C	MM	M	46	Respiratory tract
4 C	MM	M	82	Respiratory tract
5 C	MM	F	60	Respiratory tract
6 C	MM	F	68	Digestive tract
1D	HM	F	45	Genital tract
2D	HM	M	56	Digestive tract
3D	HM	F	33	Genital tract
4D	HM	F	41	Genital tract
5D	HM	F	U	Genital tract
6D	HM	M	62	Respiratory tract
7D	HM	M	38	Digestive tract
8D	HM	M	71	Digestive tract
9D	HM	F	33	Genital tract
10D	HM	F	78	Genital tract
11D	HM	F	83	Digestive tract
12D	HM	F	48	Digestive tract
13D	HM	M	NA	Digestive tract
14D	HM	M	NA	Digestive tract
15D	HM	M	NA	Digestive tract

Abbreviations: F, female; HM, healthy mucosa; M, male; MM, mucosal melanoma; NA, not available.

Previous studies exploring the association between viral infections and both CM and MM are limited and highly heterogeneous.[6–14] These investigations have mainly focused on the nucleic acid detection of herpesviruses, polyomaviruses, and papillomaviruses (usually without subsequent typing of detected HPVs) in cancer biopsies. Furthermore, since different molecular methods characterized by different sensitivities have been used, it is difficult to compare the results obtained in the different studies. Overall, in the above-referenced studies, robust evidence for a strong causal relationship between viral infections and melanomas is lacking.[6–14]

To further explore the possible association between viral infections and MM pathogenesis, in the current study, we investigated the prevalence of DNA of a broad range of viruses in 31 MMs and in 15 biopsies of healthy mucosa (HM), using molecular methods. The viruses included in this study were some oncogenic viruses: α- and β-human papillomaviruses (HPVs); Merkel cell polyomavirus (MCPyV); Epstein Barr virus (EBV); human herpesvirus 8 (HHV8); and five viruses frequently detected in cancer specimens, thus hypothetically involved in the carcinogenesis: herpes simplex virus 1 and 2 (HSV1 and HSV2), human herpesvirus 6, (HHV6), cutavirus (CutaV), and human parvovirus B19 (B19V).

2 | MATERIALS AND METHODS

2.1 | Tissue samples and patients

Formalin-fixed paraffin-embedded (FFPE) samples of primary MM tissues (n = 39) and biopsies from HM (*n* = 15) were retrospectively selected from the Archive of the Section of Pathology, Department of Health Sciences, University of Florence, Florence, Italy. Ethical Committee approval for the study was obtained from the Ethics Committee of Area Vasta Centro (*Azienda Ospedaliero Universitaria Careggi*, Florence, Italy), nr. 2015/00037100; amendment 2017-389.

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Thirty-one out of 39 MM specimens and 15 out of 15 HM samples had amplifiable DNA, since they were positive for housekeeping AP3B1 (Adaptor Related Protein complex 3 subunit Beta) gene by PCR, and these samples were tested further for the presence of viral DNA.

The amplifiable MM samples were collected from 21 females (age range 35–98 years; median age 74) and from 10 males (age range 46–87 years; median age 73). Thirteen specimens were taken from the digestive tract (9 anorectal, 1 esophagus, 2 colon, and 1 stomach), nine from the respiratory tract and oral cavity (4 nasal cavity, 4 maxillary site, 1 palate mucosa), and nine from female genital tract (vulva).

The 15 biopsies of HM were obtained from 8 females (age range 33–83 years; median age 47) and from seven males (age range 38–71 years; median age 59). Among control samples, six were collected from the female genital tract (vulva), eight from the digestive tract (3 anorectal and 5 colon), and one from the respiratory tract (nasal mucosa). Table 1 shows the characteristics of patients and samples included in the present study.

2.2 | DNA extraction

Extraction and purification of DNA from FFPE of MM and HM samples was carried out using QIAamp DNA FFPE Tissue kit, (Qiagen Valencia). Tissue sections 10 µm in thickness, were obtained from paraffin blocks for each MM and HM sample. The extraction of nucleic acids was performed according to the manufacturer's instructions and DNA was eluted with 50 µl of elution buffer. To check DNA extraction and amplification steps, all samples were analyzed by real-time PCR for the Adaptor Related Protein Complex3 Subunit-Beta-1 sequence, AP3B1 (Bio-Rad).[15]

2.3 | Viral DNA detection

To detect DNA of HSV1 and 2, EBV, HHV6 and 8, B19V, CuV, and MCPyV, PCR amplification assays were used as described in previous reports (Table 2). To detect HSV1 and EBV DNA two SybrGreen Real-time PCRs were carried out using reaction mix containing 400 nM of each primer. After the initial activation step, 40 cycles of amplification (95°C for 15 s, 60°C for 30 s, and 60°C for 10 s) were performed, with acquisition for 10 s at 60°C, after the extension step. The specificity of Sybr-Green real-time PCRs was verified by melting curve analysis. For Melting analysis, ramp from 65°C to 95°C was used, rising by 0,1°C each step.

To detect HSV2, HHV6, HHV8, B19V, CuV, and MCPyV TaqMan Real-Time PCRs were done using master mix containing 400 nM of each primer and 200 nM of probe. After initial activation step, 40 cycles of amplification (95°C for 15 s and 60°C for 30 s) were performed with acquisition during the extension step. The amplification reactions were performed using Rotor Gene Q (Qiagen).

The analytical sensitivity of the PCRs for detection of viral DNA, in our experimental conditions, was determined by endpoint titration of viral target sequences. As reported in Table 2, the sensitivity of the reactions used in this study varied from 100 to 550 genome copies/ml.

HPV detection and genotyping were carried out using line probe assays, based on the PCR amplification and reverse hybridization principle. In particular, after amplification with biotinylated primers, the biotinylated amplicons were hybridized with a type-specific oligonucleotide probes immobilized as parallel lines on membrane strip and detected with streptavidin-conjugated alkaline phosphate and BCIP/NBT chromogen. The bands were then analyzed against a reference sheet. The mucosal α-HPVs were detected and typed using Ampliquality HPV-type Express v3.0 kit (AB Analitica), which amplify a sequence localized in L1 region of viral genome and can detect 40 genotypes (Table 3). The skin β-HPVs were detected and typed using RHA Kit Skin (beta) HPV (Diassay) which amplify 117 bp sequence localized in E1 region of viral genome and is able to identify 25 genotypes (Table 3).

2.4 | Statistical analysis

Continuous variables are reported with median and range (min-max values). Given the limited number of samples, we used the exact Fisher test for the categorical variables, while for the continuous

TABLE 2 Amplification assays used to detect viral DNA.

Virus	PCR	Gene	Amplicon (pb)	Genome copies/mL	References
HSV1	Sybr-Green real-time	UL30	101	100	[16]
HSV2	TaqMan real-time	US4	72	200	[13]
EBV	Sybr-Green real-time	Polymerase	158	300	[16]
HHV6	TaqMan real-time	E67	76	200	[13]
HHV8	TaqMan real-time	ORF26	67	200	[13]
B19V	TaqMan real-time	NS1	154	550	[17]
CuV	TaqMan real-time	VP2	91	500	[18]
MCPyV	TaqMan real-time	sT	70	100	[15]

Abbreviations: B19V, human parvovirus B19; CuV, cutavirus; EBV, Epstein Barr virus; HHV, human herpesvirus; HSV, herpes simplex virus; MCPyV, Merkel cell polyomavirus; PCR, polymerase chain reaction.

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variables a nonparametric approach was used: Mann–Whitney test or Wilcoxon test for comparisons between two groups. The Odds Ratio with the 95% confidence interval (CI95%) is reported.

TABLE 3 HPV types detectable in the present study.

Assay	HPV types
AMPLIQUALITY HPV-TYPE EXPRESS (AB-Analytica)	α-HPV Species 1: HPV42 Species 3: HPV61, 62, 72, 81, 83, 84, 87, 89 Species 5: HPV26, 51, 69, 82 Species 6: HPV53, 56, 66 Species 7: HPV18, 45, 59, 68 (a and b), 70 Species 8: HPV40, 43 Species 9: HPV16, 31, 33, 35, 39, 52, 58, 67 Species 10: HPV6, 11, 44, 55 Species 11: HPV64, 73 Species 13: HPV54 Species 15: HPV71, 90
RHA KIT SKIN (Beta) HPV (LBP)	β-HPVs Species 1: HPV5, 8, 12, 14,19, 20, 21, 24, 25, 36, 47, 93 Species 2: HPV9,15, 17, 22, 23, 37, 38, 80 Species 4: HPV92 Secies 5: HPV96

Abbreviation: HPV, human papillomavirus.

The beta-HPV species (from 1 to 5) were analyzed both as categorical variable (presence/absence for each species) and as continuous variable. For the first analysis, a sample was deemed positive for a viral species when at least one virus belonging to that species was found in that specific sample. For the latter case, for each sample (MM patients or healthy donors), we report the number of identified genotypes among those tested for each single species and for all the species grouped together.

The statistical analysis was performed using the R software version 4.2.2-“Innocent and Trusting” (R Core Team (2022). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/) with RStudio.

3 | RESULTS

3.1 | Viral DNA detection in clinical samples

The prevalence of viral DNA in MM and HM found in the present study is reported in Table 4. All samples (both tumors and controls) were negative for CuV, HSV1, HSV2, EBV, HHV6, and HHV8 DNA indicating that these viruses are not associated with the pathogenesis of MM. B19V DNA was detected in 4/31(12.9%) MM and in 2/15 (13.3%) HM, and the viral load was generally low in both types of samples (from 5.4×10^3 to 6.9×10^4 copies/10^6 cells for MM and from 5.8×10^4 to 6.3×10^4 copies/10^6 cell for HM) suggesting an asymptomatic, persistent infection rather than a causative role of the virus in MM pathogenesis.

TABLE 4 Viral DNA detection in MM and HM specimens.

Viral DNA	MM: *n* = 31 (%)	HM: *n* = 15 (%)	*p*-Value	OR (CI95%)
Herpesviridae				
HSV1	0 (0)	0 (0)	1	na
HSV2	0 (0)	0 (0)	1	na
EBV	0 (0)	0 (0)	1	na
HHV6	0 (0)	0 (0)	1	na
HHV8	0 (0)	0 (0)	1	na
Parvoviridae				
B19V	4 (12.9)	2 (13.3)	1	0.964 (0.119–11.951)
CuV	0 (0)	0 (0)	1	na
Polyomaviridae				
MCPyV	8 (25.8)	8 (53.3)	0.0999	0.313 (0.0689–1.341)
Papillomaviridae				
α-HPV	1 (3.2)	0 (0)	1	inf (0.012–inf)
β-HPV	22 (70.9)	8 (53.3)	0.514	1.61 (0.36–7.04)

Note: *p*-Value was calculated with Fisher's exact test. $p < 0.05$ was considered statistically significant.

Abbreviations: B19V, human parvovirus B19; CI, confidence interval; CuV, cutavirus; HHV, human herpesvirus; HM, healthy mucosa; HPV, human papillomavirus; HSV, herpes simplex virus; MCPyV, Merkel cell polyomavirus; MM, mucosal melanoma; na, not applicable; OR, odds ratio.

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TABLE 5 Prevalence of β-HPV species in MM and HM.

HPV	MM n = 31 (%)	HM n = 15 (%)	p-Value	OR (CI 95%)
HPV-β1	14 (45.2)	3 (20.0)	0.117	3.21 (0.67–21.27)
HPV-β2	19 (61.3)	8 (53.3)	0.752	1.37 (0.33–5.71)
HPV-β3	10 (32.3)	0 (0.0)	0.019	Inf (1.32–Inf)
HPV-β4	7 (22.6)	0 (0.0)	0.078	Inf (0.76–Inf)
HPV-β5	18 (58.1)	2 (13.3)	0.005	8.58 (1.54–91.33)
β-HPV	22 (71.0)	9 (60.0)	0.514	1.61 (0.36–7.04)

Note: *p*-Value was calculated with Fisher's exact test. $p < 0.05$ was considered statistically significant.
Abbreviations: CI, confidence interval; HM, healthy mucosa; HPV, human papillomavirus; MM, mucosal melanoma; OR, odds ratio.

Eight out of 31 (25%) MM and 8 out of 15 (53.3%) HM tested positive for MCPyV DNA. The difference was not statistically significant, and the viral load was extremely low in both tumors and controls (from 2×10^2 to 9×10^3 copies/10^6 cells for MM and from 1.6×10^2 to 4.8×10^3 copies/10^6 cell for HM) suggesting, also in this case, asymptomatic persistence of viral DNA, not the etiologic role of the virus in MM.

Regarding α-HPV, HR-HPV16 DNA was detected in only 1/31 (3.2%) MM samples collected from the respiratory tract (nasal cavity). Thus, such low prevalence of α-HPV infection does not support the hypothesis of a causal association between α-HPV infection and MM.

The overall prevalence of β-HPV was high in both MM and HM (22/31 [70.9%] vs. 8/15 [53.3%], respectively) and no statistical difference was found. However, MM samples containing β-HPV Species 3 and 5 were significantly more frequent compared to HM specimens (10/31 [32.3%] vs. 0/15, $p = 0.019$ and 18/31 [58.1%) vs. 2/15 [13.3%], $p = 0.005$, respectively) (Table 5).

Moreover, as reported in Table 6, the prevalence of HPV5, 20, 21, 25 belonging to Species 1, HPV15, 22, and 37 belonging to Species 2, HPV76 (Species 3), and HPV96 (Species 5) was significantly higher in MM samples compared to HM specimens ($p < 0.05$).

As shown in Figure 1A, the overall number of β-HPV types detected in MM samples was significantly higher in comparison with HM samples ($p = 0.0268$); that is, on average, the number of β-HPV types detected in MM samples (median 3 with a range between 0 and 18) was higher than that of the control samples (median 1 with a range between 0 and 3 types).

The β-HPV types more frequently detected in MM samples belonged to Species 1, 2, and 3. In fact, in the MM samples, the number of the viral types belonging to these species spread in a wide range (0–8, 0–6, and 0–3 types, respectively), while in the HM samples, viruses belonging to only two species (Species 1 and 2, range 0–3 types were detected).

Moreover, the comparison of the number of β-HPV genotypes in MM and HM revealed a statistical difference between the number of β-HPV belonging to Species 1 ($p = 0.0434$), 3 ($p = 0.0154$), and 5 types ($p = 0.00474$).

TABLE 6 Distribution of β-HPV types in MM and HM.

	MM n = 31 (%)	HM n = 15 (%)	p-Value	OR (CI 95%)
Species β1				
HPV5	10 (32.3)	0 (0.0)	0.019	Inf (1.32–Inf)
HPV8	0 (0.0)	1 (6.7)	0.326	0 (0–18.87)
HPV12	0 (0.0)	0 (0.0)	1.000	0 (0–Inf)
HPV14	0 (0.0)	0 (0.0)	1.000	0 (0–Inf)
HPV19	2 (6.5)	0 (0.0)	1.000	Inf (0.09–Inf)
HPV20	9 (29.0)	0 (0.0)	0.021	Inf (1.12–Inf)
HPV21	10 (32.3)	0 (0.0)	0.019	Inf (1.32–Inf)
HPV24	3 (9.7)	0 (0.0)	0.541	Inf (0.2–Inf)
HPV25	9 (29.0)	0 (0.0)	0.021	Inf (1.12–Inf)
HPV36	6 (19.4)	2 (13.3)	1.000	1.55 (0.23–17.76)
HPV47	1 (3.2)	0 (0.0)	1.000	Inf (0.012–Inf)
HPV93	5 (16.1)	0 (0.0)	0.157	Inf (0.46–Inf)
Species β2				
HPV9	10 (32.3)	4 (26.7)	1.000	1.3 (0.28–7.03)
HPV15	9 (29.0)	0 (0.0)	0.021	Inf (1.12–Inf)
HPV17	0 (0.0)	0 (0.0)	1.000	0 (0–Inf)
HPV22	12 (38.7)	0 (0.0)	0.004	Inf (1.78–Inf)
HPV23	4 (12.9)	1 (6.7)	1.000	2.04(0.18–109.27)
HPV37	10 (32.3)	0 (0.0)	0.019	Inf (1.32–Inf)
HPV38	1 (3.2)	1 (6.7)	1.000	0.48 (0.006–39.22)
HPV80	4 (12.9)	4 (26.7)	0.407	0.42 (0.065–2.66)
Species β3				
HPV49	5 (16.1)	0 (0.0)	0.157	Inf (0.46–Inf)
HPV75	1 (3.2)	0 (0.0)	1.000	Inf (0.012–Inf)
HPV76	10 (32.3)	0 (0.0)	0.019	Inf (1.32–Inf)
Species β4				
HPV92	7 (22.6)	0 (0.0)	0.078	Inf (0.76–Inf)
Species β5				
HPV96	18 (58.1)	2 (13.3)	0.005	8.58 (1.54–91.33)

Note: *p*-Value was calculated with Fisher's exact test. $p < 0.05$ was considered statistically significant.
Abbreviations: CI, confidence interval; HM, healthy mucosa; HPV, human papillomavirus; MM, mucosal melanoma; OR, odds ratio.

The β-HPV types belonging to Species 1, 2, and 3 were also the most frequent in the specimens obtained from females. However, in this case, the statistical comparison between MM and controls revealed a significance only for HPVs belonging to Species 5 ($p = 0{,}007$, Figure 1B). The Figure 1C shows, that in males the overall number of β-HPV types detected in MM samples (median 3.5 in a range between 0 and 16) was higher in comparison with HM samples

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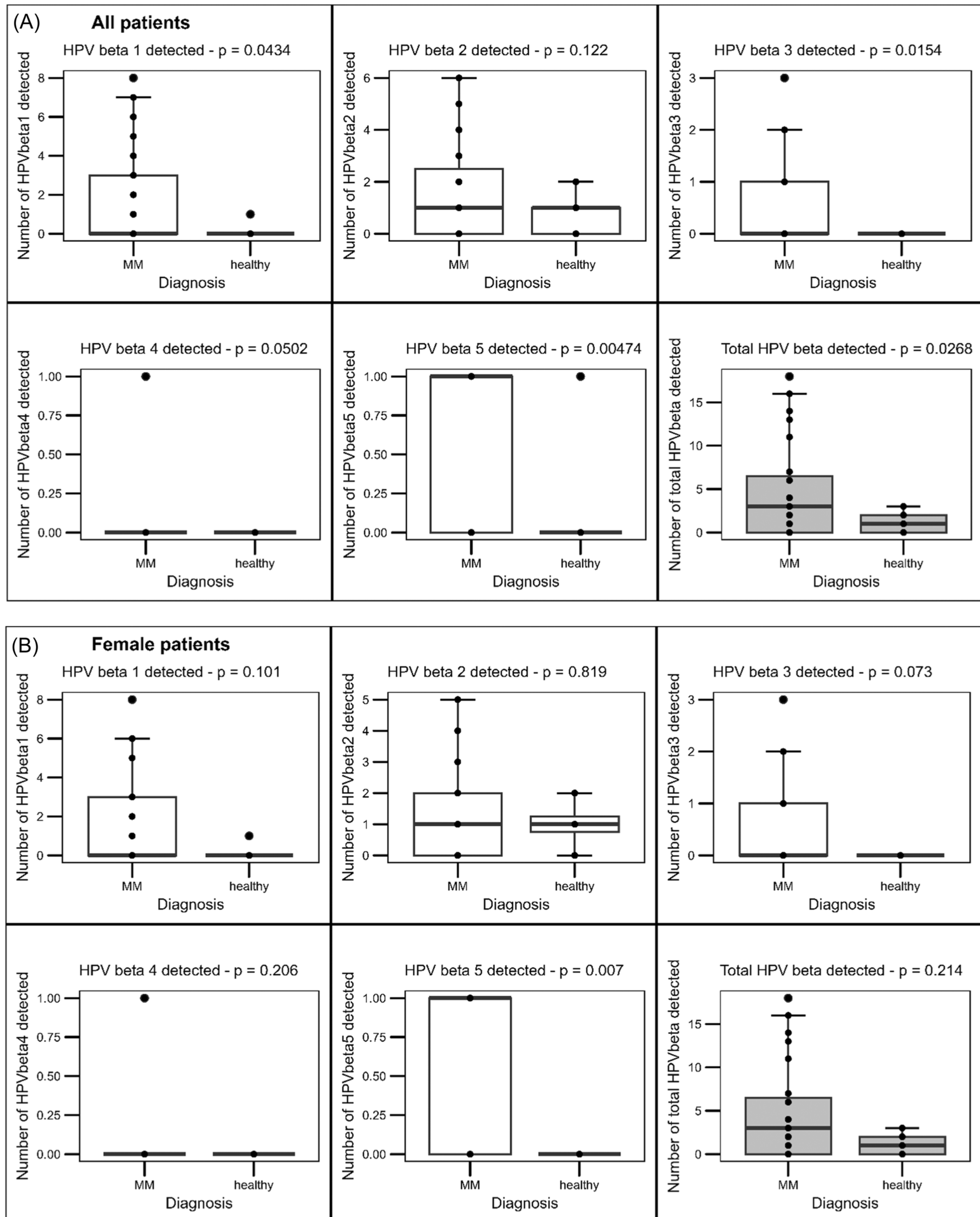

FIGURE 1 Number of β-HPV genotypes detected in MM and HM in all patients (A), in females (B), and in males (C). The number of different β-HPV genotypes and of different β-HPV species (from 1 to 5) was determined and then statistically analyzed using Wilcoxon signed rank test. The boxplot graph shows the median (black line) and the number of genotypes identified in each patient (black circles), while the whiskers were calculated using the following formula: IQR (interquartile range) × 1.5. $p < 0.05$ was considered statistically significant. HM, healthy mucosa; HPV, human papillomavirus; MM, mucosal melanoma.

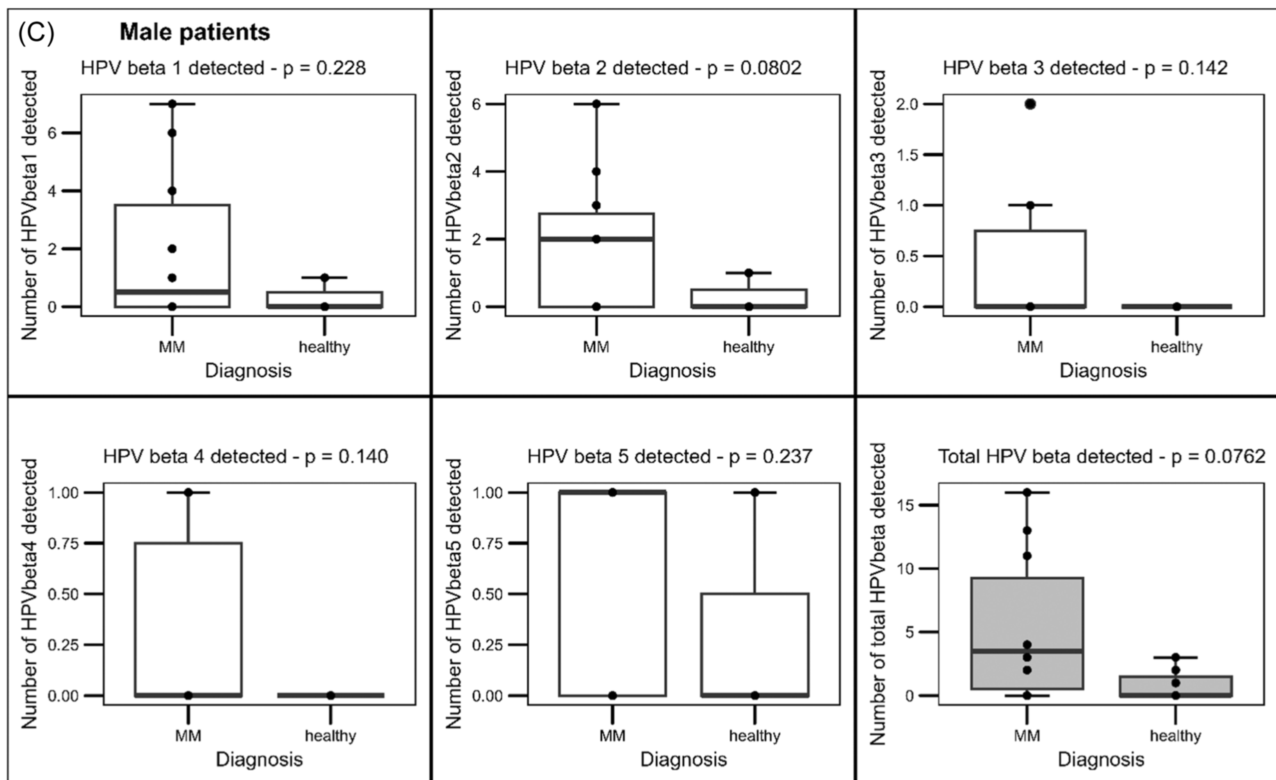

FIGURE 1 (Continued)

(median 0 with a range between 0 and 3) but no statistical significance was demonstrated.

Our results suggest that some, but not all, β-HPV types could play a role in the pathogenesis of MM.

In the digestive tract (Figure 2B), the number of HPV types was significantly higher ($p = 0.01$) in MM (median 4 with a range between 0 and 18) than in HM (median 0 with a range between 0 and 3). Moreover, the comparison of the number of β-HPV genotypes in MM and HM revealed a statistical difference between the number of β-HPV belonging to Species 2 ($p = 0.00876$), 3 ($p = 0.0319$), and 5 ($p = 0.00579$). The number of β-HPV Species 1 was also higher in MM compared with HM, but the difference did not reach statistical significance ($p = 0.0511$). There was no difference in the number of viral types in MM of the respiratory tract compared to controls ($p = 1$) (Figure 2A).

4 | DISCUSSION

To the best of our knowledge, this is the largest study investigating the presence and distribution of a broad range of DNA viruses in MMs by molecular methods. Here, we found that all MM were negative for CuV, HSV1, HSV2, EBV, HHV6, and HHV8 DNA, thus, we could exclude a role of these viruses in the pathogenesis of MM. Data in the literature are in line with our results. Indeed, the CuV genomic sequences, first detected in one sample of CM by viral enrichment and high throughput sequencing,[6] were subsequently detected in only 2 out of 185 melanoma biopsies using specific real-time PCR.[19] Some human herpesviruses were also only rarely detected in MM[13,14]

Moreover, in our study, there was no significant association between B19V infection and MM. B19V DNA was detected rarely, both in MM and HM samples, and the viral load was generally low, suggesting an asymptomatic persistence rather than a causative role of the virus in MM pathogenesis. Indeed, it is known that a low copy number of B19V DNA can persist in various organs and tissues without any apparent pathological consequences.[20]

In contrast to MCPyV-induced Merkel cell carcinoma, where viral DNA is detected in about 80% of cases[21] and the viral load is at least 1 genome per cell,[22] our results show no significant difference in the prevalence of MCPyV between MM and HM, with a very low viral load in both tumors and controls. In line with our results, a limited copy number of MCPyV genome has been demonstrated in various organs and tissues without any apparent pathological consequences.[23] Moreover, MCPyV, as well as other human polyomaviruses (PyVs) DNA, was not detected in primary MMs using sensitive Luminex assay[9] or nested type-specific PCRs.[12] Accordingly, human polyomaviruses are presumably not major factors for the development of extracutaneous melanomas.

Regarding HPV infection, it is well known that the persistence of high-risk HPV (HR-HPV) types belonging to the α-HPV genus is associated with carcinomas of the mucosal anogenital tract and head and neck carcinomas.[24] In the present study, α-HPV DNA, namely HR-HPV16 DNA, was detected in only 1 MM sample from the respiratory tract (nasal cavity). Thus, such a low prevalence of α-HPV

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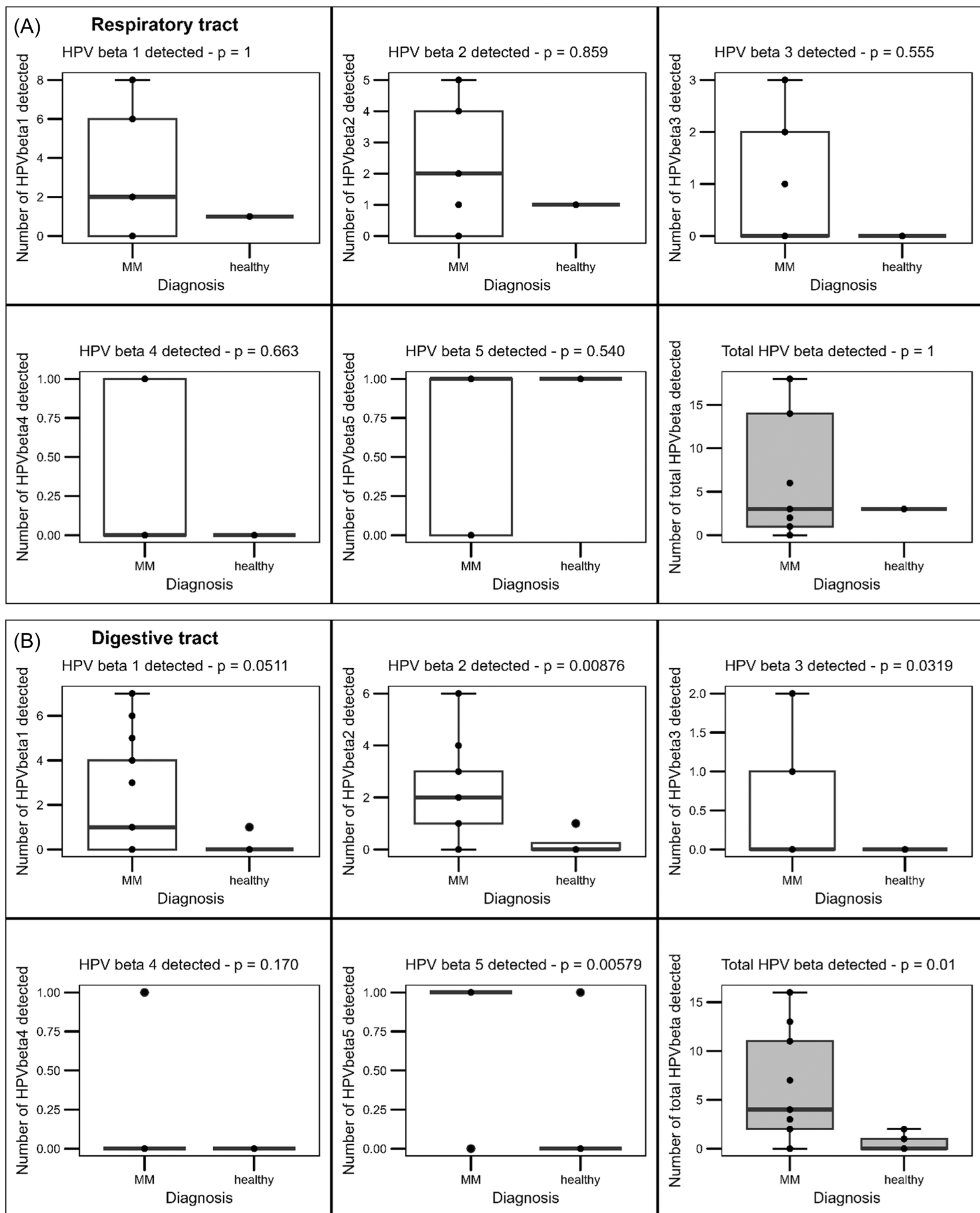

FIGURE 2 Number of β-HPV genotypes detected in MM and HM in the respiratory tract (A) or the digestive tract (B). The number of different β-HPV genotypes and β-HPV species (from 1 to 5) was detected and analyzed using Wilcoxon signed rank test. The boxplot graph shows the median (black line), the number of genotypes identified in each patient (black circles), and the whiskers calculated with the following formula: IQR × 1.5. $p < 0.05$ was considered statistically significant. HM, healthy mucosa; HPV, human papillomavirus; IQR, interquartile range; MM, mucosal melanoma.

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infection does not support the hypothesis of a causal association between α-HPV infection and MM. Moreover, our results are in line with previous studies where α-HPV DNA was not found[11] or was rarely detected in MMs.[10] However, Rohwedder and colleagues[25] describe two cases of vulvar melanomas with multiple α-HPV types, including HPV16 and some putative novel α-HPV types identified by degenerated nested PCR in both the malignant melanoma and surrounding skin. Overall, the prevalence of α-HPV reported in the literature varies from no detection to 40% positive CM[7,8,11,14] and 8% positive MM.[10] No significant association was found between melanomas and mucosal HPVs.

The β-HPV genus includes over 100 viral types classified into five distinct species: β-HPV Species 1 containing HPV5, 8,12, 14, 19, 20, 21, 24, 25, 36, 47, and 93; Species 2, containing HPV9, 15, 17, 22, 23, 37, 38, and 80; Species 3, containing HPV49, 75, and 76; Species 4, containing HPV92; and Species 5, containing HPV96.[26] β-HPVs are quite common in the skin.[27,28] They can also be found in the mucosa,[29] but their role in the pathogenesis of tumors is still unclear. Several β-HPV species and different viral types have been detected in different types of lesions[30] and seem to have varying oncogenic potential.

A group of specific β-HPV types, indicated as EV-HPVs, has been associated with cutaneous squamous cell carcinoma (SCC) in patients with Epidermodysplasia Verruciformis (EV)[29] and in organ-transplanted patients.[31] Moreover, β-HPV Species 1 types have been more frequently detected in benign skin lesions than in the matched perilesional skin, while there was a tendency for types belonging to Species 2 to be associated with SCC and actinic keratoses.[30] Patel and colleagues found that β-HPV Species 1 types were detected more often in SCC than in basal cell carcinoma (BCC).[32] In our previous investigation, we found that the genotypes belonging to Species 1 were statistically more common in non-melanoma skin cancers (NMSCs) than in paired perilesional skin, and this was also the case with BCC patients alone.[33] Using a broad spectrum of degenerate and type-specific primers, Rohwedder et al.[11] identified β-HPV DNA (both cutaneous and EV-HPV) in 67% of vulvovaginal melanomas. The authors proposed that their findings may represent a molecular record of HPV involvement in pathogenesis or progression of melanoma, which is consistent with the strong but poorly defined association of cutaneous HPV types with nonmelanoma skin cancers.

In the present study, a high prevalence of β-HPV in analyzed samples and the large number of viral types detected (especially in MM) prompted us to hypothesize a possible association between MM and β-HPV infection. Although the overall prevalence of β-HPV in MM was not significantly higher compared to controls, we found that the prevalence of β-HPV belonging to Species 3 and 5 and of some viral types belonging to Species 1, 2, 3, and 5 were significantly higher in tumors than in healthy mucosa. In addition, compared to HM, the MM samples contained a significantly higher number of β-HPV types belonging to Species 1, 3, and 5. The viral types more frequently detected in those belonging to Species 1, 2, and 3 in tumors and 1 and 2 in controls. Therefore, in line with the literature data, our results suggest that some, but not all, β-HPV types could play a role in the pathogenesis of MM. Our results, although suggesting a role for certain β-HPV types in MM oncogenesis, due to the limited sample size, require additional larger studies to support this hypothesis. Moreover, further studies are needed to investigate the biological activity of viral types detected in tumors and controls. Determination of viral load, viral gene expression, and interference of β-HPV infection with cellular gene expression in tumors and in healthy mucosa could help understand the role of these viruses in MM. Interestingly, Paolini and colleagues demonstrated that the upregulation of p16INK4a and AKT/P13K pathways in BCC is often associated with the presence of β-HPV belonging to Species 2, suggesting that in a subtype of BCC, these viruses may have a role in the carcinogenesis or in other, still undefined, biological properties of these tumors.[34]

In conclusion, the investigation of a broad range of DNA viruses, including the detection and typing of 40 mucosal and 25 cutaneous HPVs, represents a major point of the strength of this study. A potential limitation is the relatively low number of tissue samples analyzed. Overall, our data do not support a primary role for viruses in MM pathogenesis. However, some of them (mainly some β-HPV types widely detected in melanoma samples) could act as cofactors in the development of MM. Furthermore, since the MM samples contained a high number of viral types, we cannot exclude the concomitant carcinogenic action of coexisting viruses. Another possible explanation is that the viruses could persist on the mucosa as harmless commensals. Further studies are needed to understand the real significance of β-HPV DNA persistence in MM.

AUTHOR CONTRIBUTIONS

Conceptualization: Rosaria Arvia, Daniela Massi, and Krystyna Zakrzewska. *Statistic analysis*: Michele Tanturli. *Investigation and methodology*: Rosaria Arvia. *Samples selection and collection*: Filippo Ugolini and Margherita Vannucchi. *Supervision*: Daniela Massi and Krystyna Zakrzewska. *Writing—original draft preparation*: Rosaria Arvia, Daniela Massi, Michele Tanturli, Filippo Ugolini, Margherita Vannucchi, and Krystyna Zakrzewska. *Review and editing*: Daniela Massi and Krystyna Zakrzewska.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ETHICS STATEMENT

Ethical approval for the study was obtained from the Ethics Committee of Area Vasta Centro (Azienda Ospedaliero Universitaria Careggi, Florence, Italy), nr. 2015/00037100; amendment 2017-389.

ORCID

Michele Tanturli http://orcid.org/0000-0002-2642-1706

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