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## Prevaccination genomic diversity of human papillomavirus genotype 11: a study on 63 clinical isolates and 10 full-length genome sequences

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Mario Poljak

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**Prevaccination genomic diversity of human papillomavirus genotype 11: a study on 63 clinical isolates and 10 full-length genome sequences**

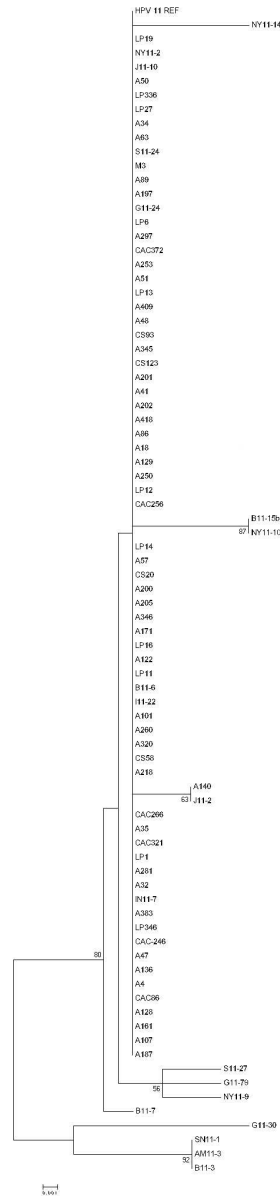
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Keywords:	human papillomaviruses, HPV 11, genomic diversity, genomic variants, complete genome

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HPV 11 (M14119)	L1 genomic positions										LCR genomic positions										E6 genomic positions					E5a genomic positions			E5b genomic positions				f											
	L1 variant	1	2	3	4	5	6	7	8	9	10	LCR variant	1	2	3	4	5	6	7	8	9	10	E6 variant	1	2	3	4	5	E5a variant	1	2	3		4	E5b variant	1	2	3	4					
Genomic variant	Reference	C	C	T	T	G	T	A	C	T	A	Reference	T	A	A	A	C	T	T	C	C	T	A	Reference	T	G	A	C	G	T	Reference	A	G	A	Reference	G	A	A	T	0				
HPV 11-1	L1-R											LCR-R												E6-1	C					E5a-R					E5b-2	C				2				
HPV 11-2	L1-R											LCR-1												E6-R						E5a-R					E5b-R					1				
HPV 11-3	L1-R											LCR-2		T										E6-1	C					E5a-R					E5b-R					2				
HPV 11-4	L1-1		T									LCR-6		C										E6-2	C		T			E5a-2	T	C			E5b-R					20				
HPV 11-5	L1-1		T									LCR-9												E6-2	C		T			E5a-2	T	C			E5b-R					5				
HPV 11-6	L1-1		T									LCR-9												E6-2	C		T			E5a-3	T	C	C		E5b-R					1				
HPV 11-7	L1-1		T									LCR-3												E6-2	C		T			E5a-3	T	C	C		E5b-R					1				
HPV 11-8	L1-1		T									LCR-3												E6-2	C		T			E5a-2	T	C			E5b-R					4				
HPV 11-9	L1-1		T									LCR-4												E6-2	C		T			E5a-2	T	C			E5b-R					1				
HPV 11-10	L1-1		T									LCR-7												E6-2	C		T			E5a-2	T	C			E5b-R					1				
HPV 11-11	L1-1		T									LCR-6												E6-3	C		T			E5a-2	T	C			E5b-R					1				
HPV 11-12	L1-1		T									LCR-6												E6-2	C		T			E5a-1	T				E5b-R					1				
HPV 11-13	L1-2		T									LCR-9												E6-2	C		T			E5a-2	T	C			E5b-R					1				
HPV 11-14	L1-3		T	G								LCR-6												E6-2	C		T			E5a-2	T	C			E5b-R					1				
HPV 11-15	L1-4		T									LCR-8		G										E6-2	C		T			E5a-2	T	C			E5b-R					1				
HPV 11-16	L1-5		T									LCR-6												E6-2	C		T			E5a-2	T	C			E5b-R					6				
HPV 11-17	L1-5		T									LCR-6												E6-2	C		T			E5a-1	T				E5b-R					1				
HPV 11-18	L1-6		T									LCR-6												E6-2	C		T			E5a-2	T	C			E5b-R					3				
HPV 11-19	L1-6		T									LCR-6												E6-4	C	A				E5a-2	T	C			E5b-R					1				
HPV 11-20	L1-7		T									LCR-10												E6-2	C		T			E5a-2	T	C			E5b-R					1				
HPV 11-21	L1-8		T									LCR-11												E6-5	C		T	A	A	E5a-2	T	C			E5b-1	A				2				
HPV 11-22	L1-9		T									LCR-5												E6-2	C		T			E5a-2	T	C			E5b-R					1				
HPV 11-23	L1-10		T									LCR-6												E6-2	C		T			E5a-2	T	C			E5b-3	C	G	C		1				
Codon position		3	5	8	1	1	2	2	2	2	2	3													1	1	2	3	1	1	1	3	3		2	4	3			3	4	4	6	6
Prototype aa		H	S	L	Y	L	A	P	R	N	S	K													S	K	I	T	P	G	L		I	V	Q		R	K	K	S	H			
Mutated as		Y	S	V	Y	L	S	P	R	N	S	T														S	K	L	T	P	E	L		F	L	P		R	Q	N	S	H		
GenBank accession numbers		FN870608-FN870750					FN870625-FN870687					FN870435-FN870497					FN870495-FN870560			FN870561-FN870623																								

Sequence variations of L1 ORF, long control region (LCR), E6, E5a, and E5b ORFs of 63 Slovenian HPV 11 isolates. At the top, genomic positions containing specific mutations are indicated vertically. Genomic positions without mutations compared to the prototype HPV 11 sequence (GenBank acc. no. M14119) are shaded in grey. Mutations in the form of nucleotide substitutions, insertions and deletions are marked with letters, I and Δ, respectively. The last column (f) indicates the number of isolates belonging to each HPV 11 genomic variant. At the bottom, codon positions and amino acid changes (aa) compared to reference HPV 11 sequence are indicated. Insertions: I1 = C; I2 = CC. 199x144mm (500 x 500 DPI)

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Neighbor-joining algorithm based phylogenetic tree of HPV 11 LCR genomic variants from Slovenia (LP19-A187), Brazil (B11-6, B11-7, B11-15b, B11-3, and AM11-3), Germany (G11-24, G11-79, and G11-30), India (IN11-7), Italy (I11-22), Japan (J11-10 and J11-2), USA-NY (NY11-2, NY11-9, NY11-10, and NY11-14), Senegal (SN11-1), and Singapore (S11-24 and S11-27). Bootstrap values (%) above 50 are shown (1000 bootstrap replicates for each grouping of a tree).

120x518mm (400 x 400 DPI)



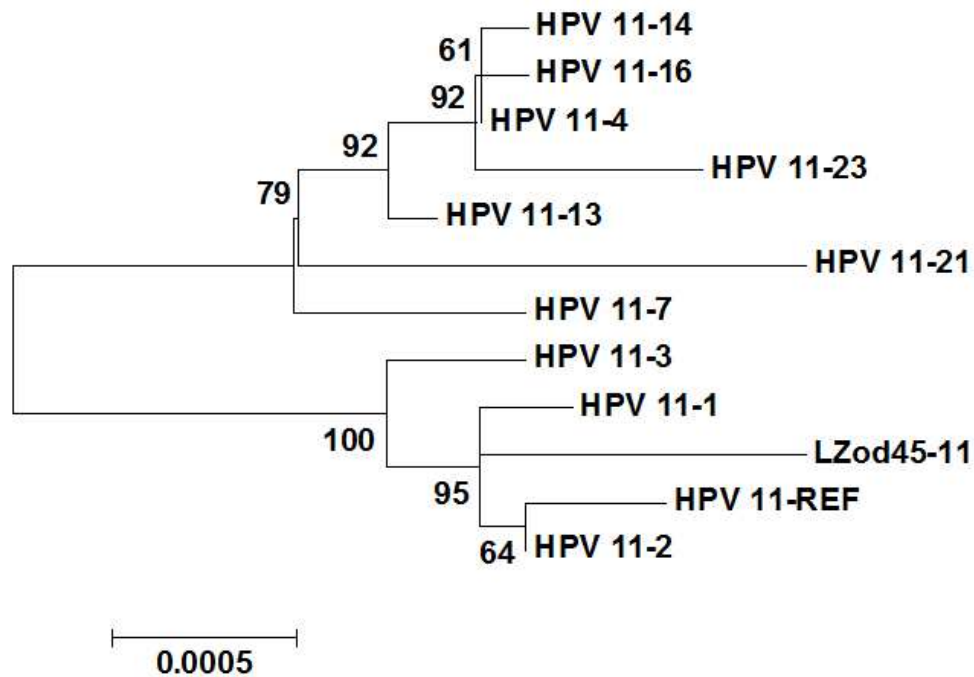


Figure 4. Neighbor-joining algorithm based phylogenetic tree of 10 complete HPV 11 genomic variants determined in this study (HPV 11-1, HPV 11-2, HPV 11-3, HPV 11-4, HPV 11-7, HPV 11-13, HPV 11-14, HPV 11-16, HPV 11-21, and HPV 11-23), prototype HPV 11 isolate (HPV 11-REF) and HPV 11 isolate LZod45 from China. Bootstrap values (%) above 50 are shown (1000 bootstrap replicates for each grouping of a tree).  
231x159mm (72 x 72 DPI)

**Table 1:** List of primers used for sequencing HPV 11 L1, LCR, E6 and E5a-E5b PCR products.

Genomic region	Primer	Sequence (5'-3')	Genomic position <sup>a</sup>
L1	HPV11-L1F	TGTTTTTATTACAGGTTCTGACTTC	5682-5706
	HPV11-L1U1	CGTAAACGTATTCCCTTATTTTTT	5743-5766
	HPV11-L1U1R	TGATCTGTTATTACCCCTTTTAC	6613-6590
	HPV6-FS3 <sup>b</sup>	GGTTGACACAGGCTTTGG	6361-6378
	HPV11-L1U2	TTTTATTTGCGAAAGGAACA	6503-6522
	HPV11-L1U2R	ACATATAAATAACACAACACACTGAC	7311-7286
	HPV11-L1R	AAAACATACATACACATTCCACAAA	7451-7427
LCR	HPV11-LCR-F	CTACAGCCCCGAAACGAA	7236-7253
	HPV11-LCR-F2	TTCGGTTGCCCTTACATACA	7598-7617
	HPV11-LCR-R	CGTGGAGGCATCTTTACTTTC	125-105
E6	HPV11-E6F	AAAATTAGCAGACGAGGCATT	81-101
	HPV11-E6R	CCACCTTGTCCACCTCATCT	650-631
E5a-E5b	HPV11-E5F	AATACCACCCACCATTAGGC	3772-3791
	HPV11-E5R	TGGCTGACGCACGTTTAC	4456-4439

<sup>a</sup> Nucleotide positions of primers were compared to the prototype HPV 11 genome (GenBank acc. no. **M14119**).

<sup>b</sup> Primer reported by Caparros-Wanderley et al. (1999).

**Table 2:** List of primers used for sequencing the long range PCR product (5400 bp) containing E7, E1, E2, E5 and L2 regions of the HPV 11 genome.

Genomic region	Primer	Sequence (5'-3')	Genomic position <sup>a</sup>
E7	11-E7F	TTGTGTGAAATAGAAAACTAAAGC	432-456
	11-E7R	TACCTGAATCGTCCGCCA	850-833
E1	11-E1F1	CTGACCTGTTGCTGTGGATG	692-711
	11-E1F2	TTACAACACAGCCAAAAAAGG	1175-1195
	11-E1F3	CGTTATTAAATATACCTGAAAATCAC	1721-1746
	11-E1F4	GGGAAGTCGTGCTTTTGC	2278-2295
	11-E1R1	ATGTTTCTCTACCTGCGTTGC	1284-1264
	11-E1R2	AAATGCCTGTCCTAAACCAATA	1813-1792
	11-E1R3	TAACTAATAACTGTTCCCCCA	2336-2315
E2	11-E1R4	CCCATTTGTTTTGCTTTGTG	2871-2852
	11-E2F1	TAGACATTGAGGATTCAGAGGAC	2693-2715
	11-E2F2	TGTAATGGAGTATGTGGTATGGA	3103-3125
	11-E2R1	CTGCCATAACATACTTCCCAAT	3291-3270
	11-E2R2	AATAAATGTAATGACATAAACCCC	3822-3799
E5	HPV11-E5F <sup>b</sup>	AATACCACCCACCATTAGGC	3772-3791
	HPV11-E5R <sup>b</sup>	TGGCTGACGCACGTTTAC	4456-4439
L2	11-L2F1	TGGTTGTTTCTGTGGTTGTTTA	4185-4206
	11-L2F2	TTTGGTGGGTTAGGTATTGGT	4573-4593
	11-L2F3	TGTCTATGAAGGAGAAGATGTAAGTT	5169-5194
	11-L2R1	TAACAGACACATCTAAAATAGCAGG	4852-4828
	11-L2R2	TGCCCAATGCGACTAAAAC	5315-5297
	11-L2R3	CTTGGATACAGGGTTGGGAG	5827-5808



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3<sup>a</sup> Nucleotide positions of primers were compared to the prototype HPV 11 genome (GenBank  
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5 acc. no. **M14119**).

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7<sup>b</sup> Primers used also for PCR amplification of the E5 HPV 11 genomic region.  
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For Peer Review

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3 1 **Prevaccination genomic diversity of human papillomavirus genotype**  
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11 4  
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22 9 Polona J. Maver<sup>1</sup>, Boštjan J. Kocjan<sup>1</sup>, Katja Seme<sup>1</sup>, Marko Potočnik<sup>2</sup>,  
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**Abstract**

Prevaccination genomic diversity of human papillomavirus genotype 11 (HPV 11) was established by sequencing 40% of the genome of 63 clinical isolates obtained from an ethnogeographically closed Caucasian cohort, and full-length genome sequencing of the ten most divergent isolates. In the study, which included the largest number of isolates to date, by analyzing pooled L1, LCR, E6, E5a, and E5b sequences (3217 bp) of an individual isolate, a total of 23 genomic variants were identified, of which three (5 isolates) and twenty (58 isolates) corresponded to prototypic and non-prototypic variant groups, respectively. Several novel, potentially important mutations are described. Full-length genome sequences of ten isolates revealed more than 99% similarity to the HPV 11 prototype isolate. The minimum genomic distance between the full-length sequences of genomic variants and the prototype was 3 point mutations and 2 inserts and the maximum distance 31 point mutations, one insertion and one deletion. Within the ethnogeographically closed cohort investigated in this study, HPV 11 was shown to be less polymorphic in comparison to the majority of HPV genotypes studied to date.

**KEYWORDS:** human papillomaviruses; HPV 11; genomic diversity; genomic variants; complete genome

**Note:** The HPV 11 nucleotide sequences reported in this paper are deposited in the DDBJ, EMBL and GenBank databases under the following accession numbers: full-length genome sequences (FN870021, FN870022, and FN907957-FN907964), L1 sequences (FN870688-FN870750), LCR sequences (FN870625-FN870687), E6 sequences (FN870435-FN870497), E5a sequences (FN870498-FN870560), and E5b sequences (FN870561-FN870623).

## 47 INTRODUCTION

48 Human papillomavirus genotype 11 (HPV 11) is one of the most prevalent HPV genotypes,  
49 classified taxonomically in the Alpha papillomavirus genus – species 10 or “HPV 6 species”,  
50 together with HPV 6, HPV 13, HPV 44, HPV 74, and PpPV1 (de Villiers et al., 2004; Bernard et  
51 al., 2010). HPV 11 was discovered in 1982 by Southern blot hybridization in a tissue specimen  
52 of a laryngeal papilloma of a 15-year-old patient (Gissmann et al., 1982). The complete  
53 nucleotide sequence of HPV 11 was determined and characterized completely in 1986  
54 (Dartmann et al., 1986). In addition to the HPV 11 prototype isolate, a full-length genomic  
55 sequence of HPV 11 isolate, obtained from a cervical swab of a patient with unspecified genital  
56 disease from China, was published in 2009 (Wu et al., 2009). Thus, to the best of our  
57 knowledge, only two full-length HPV 11 genome sequences are available at present in DDBJ,  
58 EMBL and GenBank databases (GenBank accession numbers M14119 and EU918768).

59 HPV 11 is regarded as a low-risk alpha HPV genotype since it is rarely, if ever, associated with  
60 invasive cervical cancer (Muñoz et al., 2003, 2004; Smith et al., 2007; Li et al., 2010). Next to  
61 HPV 6, HPV 11 is the major etiological agent of genital warts and laryngeal papillomas, the most  
62 frequent benign tumors in the anogenital region and lower respiratory tract of both genders,  
63 respectively (Gale et al., 1994; Potočnik et al., 2007; Aubin et al., 2008). In addition, HPV 11 is  
64 responsible for the development of approximately 4.4% of low grade squamous cell  
65 intraepithelial lesions (LSIL) (Clifford et al., 2005), and is the 18<sup>th</sup> most prevalent genotype in  
66 HPV positive women with normal cytology (de Sanjosé et al., 2007). HPV 6 and HPV 11 have  
67 been included in the quadrivalent HPV vaccine developed recently, as the most important low-  
68 risk HPV genotypes worldwide (Garland et al., 2007).

69 The genomic diversity of high-risk alpha HPV genotypes has been investigated extensively.  
70 Several studies have identified a considerable degree of viral diversity among high-risk HPV and  
71 an important role of different genomic variants in viral transmission, persistence and progression  
72 of the clinical disease (Xi et al., 1995; Da Costa et al., 2002; Burk et al. 2003; Grodzki et al.,

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3 73 2006; Sichero et al., 2007; Schiffman et al., 2010). In contrast, knowledge of the genetic  
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5 74 diversity of low-risk HPV genotypes is fairly limited. A few previous studies have focused on the  
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7 75 genomic diversity of a limited number of low-risk HPV isolates, for the most part investigating  
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10 76 only a single genomic region. The first extensive study on HPV 6 genomic diversity, analyzing  
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12 77 approximately half of the viral genome of 77 HPV 6 isolates, was only published recently (Kocjan  
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14 78 et al., 2009). To the best of our knowledge, only a single HPV 11 genomic diversity study, in  
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16 79 which 40 isolates from 8 different geographic regions were investigated across a 252 bp  
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18 80 segment of the long control region (LCR), has been published so far (Heinzel et al., 1995). Ten  
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20 81 genomic variants of HPV 11 were identified in this study.

22 82 In order to investigate further the genomic diversity of HPV 11, a total of 63 HPV 11 isolates  
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24 83 obtained from the same number of Slovenian patients were analyzed in the present study. The  
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26 84 identification and characterization of HPV 11 genomic variants was performed in all 63 isolates  
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28 85 by sequence analysis of five different genomic regions: L1, LCR, E6, E5a, and E5b, over a span  
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30 86 of 3217 bp, or approximately 40% of the viral genome. In addition, the full-length genome  
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32 87 sequence was determined for representative isolates of the 10 most divergent HPV 11 genomic  
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34 88 variants. The coding regions were examined in detail to determine the prevalence, extent and  
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36 89 distribution of amino acid changes that might have important biological implications. This study,  
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38 90 carried out on the largest number of HPV 11 isolates to date, is believed to be the first extensive  
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40 91 study of the genomic diversity of HPV 11.  
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## 46 93 **MATERIALS AND METHODS**

### 48 94 **Clinical samples**

50 95 A total of 63 HPV 11 isolates obtained from the sample collection of the Slovenian HPV  
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52 96 Reference Laboratory were available in sufficient amount to be included in the study. They were  
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54 97 obtained from the same number of epidemiologically unrelated Slovenian patients (54 males, 9  
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56 98 females). Fifty-one isolates were obtained from 42 anal swabs and 9 tissue specimens collected  
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3 99 from 48 male and 3 female patients suffering from exophytic genital warts on different parts of  
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5 100 the anogenital area, 8 isolates were obtained from tissue specimens collected from 6 male and 2  
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7 101 female patients suffering from recurrent laryngeal papillomatosis, and 4 isolates were obtained  
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9 102 from cervical swabs collected from women suffering from cervical intraepithelial neoplasia grade  
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11 103 1 lesions.  
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### 16 105 **Amplification of HPV 11 L1, LCR, E6, and E5 genomic regions**

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18 106 The genomic diversity of all HPV 11 isolates was investigated within the L1, LCR, E6, E5a, and  
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20 107 E5b regions of the HPV 11 genome. A 1770 bp fragment containing the complete L1 ORF (1506  
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22 108 bp) was amplified by PCR using HPV11-L1F (5'-TGTTTTTATTACAGGTTCTGACTTC-3', nt  
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24 109 5682-5706) and HPV11-L1R (5'-AAAACATACATACACATTCCACAAA-3', nt 7451-7427)  
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26 110 primers. An 823 bp fragment containing the complete LCR genomic region (758 bp) was  
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28 111 amplified by PCR with the primer pair HPV11-LCR-F (5'-CTACAGCCCCCAAACGAA-3', nt  
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30 112 7236-7253) and HPV11-LCR-R (5'-CGTGGAGGCATCTTTACTTTC-3', nt 125-105). A 570 bp  
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32 113 fragment containing the complete E6 ORF was amplified by PCR using the primer pair HPV11-  
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34 114 E6F (5'-AAAATTAGCAGACGAGGCATT-3', nt 81-101) and HPV11-E6R (5'-  
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36 115 CCACCTTGTCACCTCATCT-3', nt 650-631). A 685 bp fragment, which contained both E5a  
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38 116 and E5b ORF (276 and 225 bp) was PCR amplified using the primer pair HPV11-E5F (5'-  
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40 117 AATACCACCCACCATTAGGC-3', nt 3772-3791) and HPV11-E5R (5'-  
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42 118 TGGCTGACGCACGTTTAC-3', nt 4456-4439). All primers used in the study were designed  
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44 119 according to the prototype HPV 11 genome sequence (GenBank acc. no. M14119) using  
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46 120 Primer3 (<http://frodo.wi.mit.edu/>) and Netprimer (<http://www.premierbiosoft.com>) programs. A  
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48 121 corrected LCR sequence with the insertion of 2 bp at nt 7717-7718 (Heinzel et al., 1995) was  
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50 122 used for designing and genomic position numbering of the LCR specific primers.  
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55 123 All PCRs were carried out on the GeneAmp PCR instrument 9700 (PE Applied Biosystems,  
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57 124 Foster City, USA) using FastStart High Fidelity PCR System (Roche Diagnostics, Mannheim,  
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3 125 Germany). The reaction mixture was prepared in 0.2 ml reaction tubes, each containing up to  
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5 126 125 ng of template DNA, 2.5  $\mu$ l of 10 x FastStart High Fidelity Reaction Buffer with 18 mM  
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7 127  $MgCl_2$ , 200  $\mu$ M of dNTPs, 1.25 U of FastStart High Fidelity Enzyme, 0.4  $\mu$ M of each of the gene-  
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9 128 specific primers and water up to 25  $\mu$ l. The cycling conditions used were 95°C for 5 min,  
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11 129 followed by 40 cycles of 95°C for 30 s, 52-54°C for 30 s and 72°C for 1 min. This was followed  
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13 130 by a final extension step at 72°C for 7 min and cooling of reaction mixtures to 4°C.  
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### 18 132 **Sequencing and identification of HPV 11 genomic variants**

20 133 The PCR products were analyzed on ready-to-use PCR CheckIT Wide Mini S-2x25 gels  
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22 134 (Elchrom Scientific, Zurich, Switzerland) using a 100 or 500 bp DNA ladder (Roche Diagnostics,  
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24 135 Mannheim, Germany) and purified with a QIAquick PCR purification kit (Qiagen, Hilden,  
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26 136 Germany). Concentrations of purified PCR products were estimated on a 0.8% agarose gel  
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28 137 using a MassRuler DNA Ladder Low Range (Fermentas, Vilnius, Lithuania) and set to 50 ng/ $\mu$ l.  
29  
30 138 Sequencing of PCR products was performed at Macrogen. (Seoul, Korea) with the same set of  
31  
32 139 primers as those used for PCR and additional sequencing primers that are listed in Table 1. All  
33  
34 140 sequences were processed and assembled with Vector NTI Advance v11.0 (Invitrogen,  
35  
36 141 Carlsbad, CA). The identification of HPV 11 L1, LCR, E6, E5a, and E5b genomic variants was  
37  
38 142 made with BioEdit Sequence Alignment Editor v7.0.9.0 (North Carolina State University,  
39  
40 143 Raleigh, USA) and the sequence of prototype HPV 11 genome (GenBank acc. no. M14119) was  
41  
42 144 used as a standard for comparisons and genomic position numbering. The corrected LCR  
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44 145 sequence of prototype HPV 11 genome was used to determine HPV 11 LCR genomic variants  
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46 146 (Heinzel et al., 1995). In order to minimize PCR and sequencing errors, all isolates that  
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48 147 represented a single HPV 11 genomic variant were amplified and sequenced twice. According to  
49  
50 148 the presence of specific fixed nucleotide signatures, the genomic variants were grouped into  
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52 149 "prototypic" and "non-prototypic" HPV 11 genomic variant groups.  
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## 151 **Full-genome amplification and sequencing of selected HPV 11 isolates**

152 Full-length genome sequencing was performed on a total of 10 HPV 11 isolates: 5 isolates were  
153 obtained from anal swabs of male patients suffering from exophytic genital warts (variants HPV  
154 11-1, HPV 11-3, HPV 11-7, HPV 11-13, HPV 11-16), 2 isolates from tissue specimens of male  
155 patients suffering from exophytic genital warts (variants HPV 11-14, HPV 11-21), 2 isolates from  
156 tissue specimens of male patients suffering from laryngeal papillomatosis (variants HPV 11-4,  
157 HPV 11-23) and one isolate from a cervical swab of a female patient with a cervical  
158 intraepithelial neoplasia grade 1 lesion (variant HPV 11-2).

159 Two overlapping DNA fragments (2900 and 5400 bp), covering the complete HPV 11 genome,  
160 were generated by PCR using two pairs of primers. The 2900 bp fragment was generated using  
161 11-L1F-PCR (5'-TTTATTACAGGTTCTGACTTCTATTTGC-3', nt 5686-5713) and 11-E6R-PCR  
162 (5'-CCACCTTGTCCACCTCATCTTC-3', nt 650-629) primers and the 5400 bp fragment was  
163 generated using 11-E7F-PCR (5'-CGTTGTGTGAAATAGAAAACTAAAGC-3', nt 430-456) and  
164 11-L2R3-PCR (5'- ATACAGGGTTGGGAGGAGGC-3', nt 5822-5803) primers. Both PCR  
165 reactions were performed using Expand Long Template PCR System (Roche Diagnostics,  
166 Manheim, Germany).

167 The reaction mixture contained up to 500 ng of template DNA, 2.5 µl of Expand Long Template  
168 buffer 2 with MgCl<sub>2</sub>, 500 µM of dNTPs, 0.75 U of Expand Long Template Enzyme mix, 0.3 µM of  
169 each of the gene-specific primers, and water up to 25 µl. The cycling conditions used were 94 °C  
170 for 2 min, followed by 40 cycles of 94 °C for 10 s, 52 °C for 30 s and 68 °C for 3-5 min. This was  
171 followed by a final extension step at 68 °C for 7 min and cooling of reaction mixtures to 4 °C.

172 Sequencing of the long range PCR products was performed at Macrogen. (Seoul, Korea) with  
173 primers listed in Tables 1 and 2. All sequences were edited and assembled with Vector NTI  
174 Advance v11.0 (Invitrogen, Carlsbad, CA) and processed for variant identification in the same  
175 manner as described above. In addition, the L1-LCR-E6 genomic fragments (2900 bp) were  
176 compared to our previous sequencing results of L1, LCR and E6 regions of the selected isolates



177 using BioEdit Sequence Alignment Editor v7.0.9.0, in order to confirm the accuracy of our  
178 previous work.

179  
180 **Phylogenetic analyses**  
181 All phylogenetic analyses were performed using the MEGA4.0.1 program package, as described  
182 previously (Tamura et al., 2007). Neighbor-joining, minimal evolution, UPGMA and maximum  
183 parsimony algorithms were used to construct the phylogenetic trees.

## 185 **RESULTS**

186 All 63 HPV 11 isolates included in the study were amplified successfully and sequenced across  
187 the entire L1 (nt 5682-7451), LCR (nt 7236-125), E6 (nt 81-650) and E5a-E5b (nt 3772-4456)  
188 genomic regions. The HPV 11 L1, E6, E5a and E5b genomic variants were identified using the  
189 prototype HPV 11 genome (GenBank acc. no. M14119) as a standard for comparisons and  
190 nucleotide position numbering. For the determination of HPV 11 LCR genomic variants, an LCR  
191 sequence of the prototype HPV 11 amended by the inclusion of two base pairs (GC) following a  
192 G at position 7716 (Heinzel et al., 1995) was used.

### 194 **HPV 11 L1 genomic variants**

195 Among 63 HPV 11 isolates included in the study, a total of eleven L1 genomic variants, including  
196 the prototype L1 sequence, were identified (Fig. 1). Five of the 63 (7.9%) isolates corresponded  
197 to the prototype L1 sequence. Point mutations were observed in 11 genomic positions, with a  
198 maximum genomic distance of 3 nucleotides between the variants and the prototype HPV 11  
199 sequence (0.2% of the entire L1 ORF). Nucleotide substitutions altered the amino acid  
200 sequence of the L1 protein by a maximum of one amino acid (0.2% of the L1 protein) in four L1  
201 genomic variants (L1-2, L1-3, L1-5 and L1-10) represented by 10 isolates (one, one, seven and  
202 one isolate for each of the variants, respectively) (Fig. 1).

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5 204 **HPV 11 LCR genomic variants**

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7 205 Among 63 HPV 11 isolates, a total of twelve LCR genomic variants, including the prototype LCR  
8 206 sequence, were identified (Fig. 1). Two of the 63 (3.2%) isolates corresponded to the prototype  
9 207 LCR sequence. Mutations were observed in 11 genomic positions and included 9 nucleotide  
10 208 substitutions, one 1 bp (T) deletion, one 1 bp (C) insertion and one 2 bp (CC) insertion. The  
11 209 maximum genomic distance between the variants and the prototype sequence was 6 mutations  
12 210 (affecting 0.8% of the complete LCR region) (Fig. 1).  
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23 212 **HPV 11 E6 genomic variants**

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25 213 Among 63 HPV 11 isolates, a total of six E6 genomic variants, including the prototype E6  
26 214 sequence, were identified (Fig. 1). One of the 63 (1.6%) isolates corresponded to the prototype  
27 215 E6 sequence. Point mutations were observed in 7 genomic positions and the maximum genomic  
28 216 distance between the variants and the prototype sequence was 5 nucleotides (1.1% of the entire  
29 217 E6 ORF). Nucleotide substitutions altered the amino acid sequence of the E6 protein by a  
30 218 maximum of two amino acids (1.3% of the E6 protein) in the E6-5 genomic variant, represented  
31 219 by two isolates (Fig. 1).  
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42 221 **HPV 11 E5a genomic variants**

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44 222 Among 63 HPV 11 isolates, a total of four E5a genomic variants, including the prototype E5a  
45 223 sequence, were identified (Fig. 1). Five of the 63 (7.9%) isolates corresponded to the prototype  
46 224 E5a sequence. Point mutations were observed in 3 positions and the maximum genomic  
47 225 distance between genomic variants and the prototype sequence was 3 nucleotides (1.1% of the  
48 226 entire E5a ORF). Nucleotide substitutions altered the amino acid sequence of the E5a protein by  
49 227 one, two or three amino acids (up to 3.3% of the E5a protein) in three E5a genomic variants  
50 228 (E5a-1, E5a-2 and E5a-3), represented by 58 isolates (two, fifty-four and two isolates) (Fig. 1).  
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**HPV 11 E5b genomic variants**

Among 63 HPV 11 isolates, a total of four E5b genomic variants, including the prototype E5b sequence, were identified (Fig. 1). Fifty-eight of 63 (92.1%) isolates corresponded to the prototype E5b sequence. Point mutations were observed in 5 genomic positions. The maximum genomic distance between the variants and the prototype sequence was 3 nucleotides (1.3% of the entire E5b ORF). Nucleotide substitutions altered the amino acid sequence of the E5b protein by a maximum of one amino acid (1.3% of the E5b protein) in two E5b genomic variants (E5b-2 and E5b-3), represented by two and one isolate (Fig. 1).

238

**HPV 11 genomic variants**

The obtained nucleotide data of L1, LCR, E6, E5a, and E5b genomic regions were combined for an individual isolate and HPV 11 genomic variants were determined. Among 63 analyzed HPV 11 isolates, a total of 23 genomic variants were identified (Fig. 1) but, surprisingly, none of them corresponded to the prototype HPV 11 sequence. The minimum variant divergence from the prototype sequence was identified in HPV 11-2 genomic variant (one 1 bp insertion) and the maximum variant divergence was found in HPV 11-21 genomic variant (13 point mutations, one 1 bp deletion and one 2 bp insertion) (Fig. 1). As shown in Fig. 1, a strong intergenomic co-variation, grouping all analyzed HPV 11 isolates into "prototypic" and "non-prototypic" HPV 11 genomic variant groups, was observed between all analyzed genomic regions except E5b ORF. The prototypic group consisted of five (7.9%) HPV 11 isolates (3 genomic variants) and the non-prototypic group consisted of 58 (92.1%) HPV 11 isolates (20 genomic variants).

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**Phylogenetic analysis of the HPV 11 LCR**

Sequence comparison of a 252 bp segment of the LCR region (nt 7625-7876) of 40 isolates from different parts of the world (Heinzel et al., 1995) and of 63 isolates from the present study,

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3 255 revealed that the prototype LCR sequence is the most abundant genomic form present in 26/40  
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5 256 and 62/63 analyzed samples, respectively. One LCR genomic variant found in our study  
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7 257 corresponded to the J11-2 HPV 11 LCR variant from Japan, identified by Heinzl et al. (1995).  
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10 258 Phylogenetic analysis of a 252 bp segment of the LCR of: (i) 63 Slovenian HPV 11 isolates, (ii)  
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12 259 the prototype HPV 11 sequence and (iii) 19 isolates of HPV 11 originating from 8 different  
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14 260 geographic regions (Brazil, Germany, India, Italy, Japan, USA-NY, Senegal and Singapore) is  
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16 261 shown in Fig. 2.  
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### 21 263 **HPV 11 full-length genome sequencing and phylogenetic analysis**

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23 264 One representative of each of the prototypic HPV 11 genomic variants (HPV 11-1, HPV 11-2  
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25 265 and HPV 11-3), one representative of six non-prototypic HPV 11 genomic variants (HPV 11-7,  
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27 266 HPV 11-13, HPV 11-14, HPV 11-16, HPV 11-21 and HPV 11-23) containing specific amino acid  
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29 267 changes in L1, E6, and/or E5 coding regions, and one representative of the most common HPV  
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31 268 11 genomic variant (HPV 11-4), also belonging to the non-prototypic group, were selected for  
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33 269 full-length genome sequencing, additionally to determine the level of genomic variation in  
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35 270 previously non-analyzed genomic regions (E7, E1, E2/E4, and L2), and to perform the first  
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37 271 phylogenetic analysis of HPV 11 full-length genome sequences. Among the 10 selected HPV 11  
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39 272 isolates, two E7, eight E1, four E2/E4 and six L2 genomic variants were identified (Fig. 3). The  
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41 273 minimum genomic distance between the full-length genome sequences of genomic variants and  
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43 274 the HPV 11 prototype was 3 point mutations and 2 inserts (HPV 11-2) and the maximum  
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45 275 genomic distance was 31 point mutations, one insertion and one deletion (HPV 11-21). Even the  
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47 276 isolate with the maximum genomic distance showed more than 99% similarity to the prototype  
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49 277 sequence. A strong intergenomic co-variation was also observed in the E2/E4, E7 and L2  
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51 278 genomic regions. Phylogenetic analysis of the 10 full-length HPV 11 genome sequences (7932-  
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53 279 7943 bp) determined in this study and both full-length HPV 11 genome sequences available  
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55 280 previously in GenBank (acc. nos. M14119 and EU918768) confirmed the grouping of HPV 11  
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3 281 isolates (genomic variants) into two closely related clusters. As shown in Fig. 4, one cluster  
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5 282 contains the prototype HPV 11 isolate, isolate LZod45-11 (Wu et al., 2009) and the three  
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7 283 prototypic HPV 11 genomic variants HPV 11-1, HPV 11-2, and HPV 11-3. The second cluster  
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10 284 contains seven non-prototypic HPV 11 genomic variants: HPV 11-4, HPV 11-7, HPV 11-13, HPV  
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12 285 11-14, HPV 11-16, HPV 11-21 and HPV 11-23.

13 286

16 287 **DISCUSSION**

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18 288 HPV 11 prevaccination genomic diversity is described over a span of 3217 nucleotides  
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20 289 (approximately 40% of the viral genome) among the highest number of HPV 11 isolates to date.  
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22 290 To the best of our knowledge, this work is the first investigation of genomic diversity of the four  
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24 291 important genomic regions of HPV 11: L1, E6, E5a, and E5b. By pooling the nucleotide data of  
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26 292 five different genomic regions for each of the 63 isolates included in this study, a total of 23  
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28 293 different HPV 11 genomic variants were identified. Nucleotide signatures specific for the  
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30 294 prototypic and non-prototypic HPV 11 genomic variant lineages were clearly identified in all  
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32 295 genomic regions studied except E5b ORF; these regions showed, as a main feature, fixed  
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34 296 nucleotide variations in at least one position. The presence of specific nucleotide signatures  
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36 297 scattered through the whole genome was confirmed after full-length genome sequencing of the  
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38 298 ten most divergent genomic variants. In addition, full-length sequencing allowed the first  
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40 299 investigation of HPV 11 genomic diversity in E7, E1, E2/E4 and L2 genomic regions.

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42 300 The L1 protein is the major capsid protein of HPV and, during natural infection, it is the main  
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44 301 target of neutralizing antibody responses. In addition, the quadrivalent prophylactic HPV vaccine  
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46 302 against HPV 6, HPV 11, HPV 16, and HPV 18 is produced by expression of the L1 gene as a  
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48 303 recombinant protein, capable of self-association and formation of virus-like particles (VLPs) that  
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50 304 morphologically resemble native capsids, inducing a strong immune response (Bryan 2007). Any  
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52 305 alterations of the L1 amino acid sequence may thus have an impact on conformation-dependent  
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54 306 epitopes and, consequently, on the antigenic properties of the L1 viral protein. A few binding  
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3 307 sites within the HPV 11 L1 protein have previously been identified, and the effect of amino acid  
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5 308 substitutions in these regions on a reduction or loss of binding capacity of neutralizing antibodies  
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7 309 has been reported (Ludmerer et al., 1996, 1997, 2000). A total of 11 L1 genomic variants were  
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9 310 identified in the present study, of which four, represented by 10 HPV 11 isolates, contained  
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11 311 specific amino acid substitutions in L1 protein. The L1 genomic variants had specific amino acid  
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13 312 substitutions (H35Y, L119V, A235S or K347T) at nucleotide positions 5873, 6125, 6473 or 6810,  
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15 313 respectively. The single amino acid change A235S was the most frequent mutation, being found  
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17 314 in 7/63 (11.1%) HPV 11 isolates. The biological and clinical significance of the majority of these  
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19 315 mutations is at present unclear and needs to be investigated further. To the best of our  
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21 316 knowledge, only the K347T is located in a domain responsible for elicitation of a neutralizing  
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23 317 response to HPV 11 (Ludmerer et al., 2000).

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27 318 Sequence variations within the LCR region could theoretically influence the binding affinity of the  
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29 319 different transcription factors and thus affect viral transcription and replication (Sichero and Villa  
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31 320 2006). A total of 12 LCR genomic variants were identified in this study, and all clearly separated  
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33 321 into prototypic or non-prototypic HPV 11 variant groups. An LCR sequence completely identical  
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35 322 to the HPV 11 prototype was found in only 2 of our isolates, while the maximum genomic  
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37 323 difference between the sequences of the prototype and the newly identified LCR variants was 6  
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39 324 mutations. Of these, a nucleotide substitution at position 7479 and a nucleotide deletion at  
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41 325 position 7509 were found to be characteristic for all non-prototypic HPV 11 genomic variants. In  
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43 326 addition, the inclusion of two base pairs (GC) following a G at position 7716 in the prototype  
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45 327 HPV 11 sequence determined by Heinzl et al. (1995) was confirmed, since it was found in all  
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47 328 63 isolates studied. In contrast to the findings of Heinzl et al. (1995), no inserts longer than 2  
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49 329 bp were found in the LCR region in any of our isolates.

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53 330 In contrast to the well established roles of high-risk HPV E6 proteins in cellular transformation  
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55 331 and viral life cycle, the biological functions of low-risk HPV E6 proteins remain largely unclear. It  
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57 332 has been shown, however, that HPV 11 E6 and E7 proteins play an important role in the viral  
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3 333 productive life cycle by facilitating the episomal maintenance of the viral genomes, and may act  
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5 334 in a manner similar to that observed for the proteins of high-risk HPVs (Oh et al., 2004). Thus,  
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7 335 even a single mutation in the HPV 11 E6 protein sequence can affect dramatically its function  
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10 336 (Oh et al., 2004). Among six E6 genomic variants identified in this study, only one (Fig.1, variant  
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12 337 E6-5), represented by two HPV 11 isolates, contained specific amino acid substitutions in the E6  
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14 338 protein: I92L (nt 186) and G122E (nt 466). Both mutations identified in our study were different  
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16 339 from those previously described as impairing episomal maintenance (Oh et al., 2004).  
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18 340 Interestingly, I92L and G122E have been described previously in the HPV 11VC genomic  
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20 341 variant detected in penile verrucous carcinoma (McGlennen et al., 1992). In cell transformation  
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22 342 assays, this genomic variant transformed neonatal rat kidney epithelial cells with an efficiency  
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24 343 comparable to HPV 16 (McGlennen et al., 1992). Characterization of the immunologic responses  
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26 344 elicited by the experimental therapeutic DNA vaccine that targets the HPV 11 E6 gene, identified  
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28 345 E6<sub>aa44-51</sub> peptide as the most immunogenic region for HPV 11 (Peng et al., 2010). According to  
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30 346 the results of this study, none of the 23 HPV 11 genomic variants contained amino acid changes  
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32 347 in that particular region; the efficiency of this candidate vaccine does not therefore seem to be  
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34 348 compromised by genomic variation.

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37 349 The HPV E5 proteins are highly hydrophobic proteins found in the membrane compartments of  
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39 350 the host cells. Their physiological roles are not completely understood. It is thought that they  
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41 351 have the ability to modulate cell signaling, possess transforming activity, play a role in the  
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43 352 stimulation of cell proliferation and, together, with the HPV E6 proteins induce koilocytosis  
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45 353 (Doorbar et al., 2006; Krawczyk et al., 2008). Unlike the majority of other HPV genotypes, which  
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47 354 have one E5 ORF, HPV 11 and closely related HPV 6 possess two E5 genes: E5a and E5b. It  
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49 355 has been shown that HPV 11 E5 proteins are involved in transformation (Chen et al., 1994,  
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51 356 1996a, 1996b) and stimulation of cell proliferation (Tsao et al., 1996). The mutational analysis of  
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53 357 the HPV 11 E5a protein showed that nucleotide substitutions, deletions and insertions in the E5a  
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55 358 gene can significantly impair the E5a transforming activity (Chen et al., 1996a). In this study,  
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3 359 four E5a genomic variants were found, of which three (all non-prototypic variants), differed from  
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5 360 the amino acid sequence of the prototype HPV 11 E5a protein by at least one mutation. The  
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7 361 most frequent E5a genomic variant (Fig. 1, variant E5a-2) identified in our study contained two  
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9 362 specific amino acid substitutions (I28F and V41L) at nucleotide positions 3952 and 3991,  
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11 363 respectively. Since these two substitutions were found in the majority (56/63, 88.8%) of HPV 11  
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13 364 isolates characterized in this study, it is presumed that they prevail in the naturally occurring  
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15 365 HPV 11 population. One E5a genomic variant (Fig. 1, E5a-1) contained only the I28F amino acid  
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17 366 change and the second (Fig. 1, E5a-3) contained the Q91P amino acid change (nt 4142) in  
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19 367 addition to I28F and V41L. However, none of the observed E5a amino acid substitutions  
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21 368 corresponded to those previously shown to be able to affect E5a transforming activity (Chen et  
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23 369 al., 1996a). In contrast to E5a, the most frequently encountered E5b genomic variant  
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25 370 corresponded to the reference HPV 11 E5b sequence, which was found in 58/63 (92.1%)  
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27 371 isolates. Two other genomic variants (Fig. 1, variants E5b-2 and E5b-3) each contained amino  
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29 372 acid changes K43Q or K43N at nucleotide positions 4272 or 4274, respectively.

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31 373 The molecular analysis of full-length genome sequences of representative isolates of the 10  
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33 374 genomic variants, showing the greatest degree of genetic variability in the L1, E6, and/or E5  
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35 375 genomic regions (seven non-prototypic and three prototypic), revealed additional mutations  
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37 376 located in the regions of the HPV 11 genome outside of those investigated in the first part of the  
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39 377 study. Within E7 ORF, 7/10 HPV 11 variants (all non-prototypic) contained a specific amino acid  
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41 378 substitution A45S at nucleotide position 662. This amino acid change has not been reported  
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43 379 previously and its significance is unknown at present. Within E1 ORF, one genomic variant (Fig.  
44  
45 380 3, variant HPV 11-7) contained a specific amino acid substitution N100T at nucleotide position  
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47 381 1130 and two variants (Fig. 3, variants HPV 11-21 and HPV 11-23) each contained a specific  
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49 382 amino acid substitution (Q161K or Q161E) at nucleotide position 1312. An amino acid  
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51 383 substitution R318A at nucleotide positions 1783-1784 was identified in all ten HPV 11 genomic  
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53 384 variants examined. Since nucleotide mutations at positions 1783-1784 and the corresponding  
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3 385 amino acid substitution R318A were also found in the prototypic HPV 11 isolate LZod45-11 (Wu  
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5 386 et al., 2009), thus in 11 out of 12 HPV 11 isolates for which a full-length sequence is available at  
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7 387 present, we suggest the resequencing of these particular positions in the HPV 11 prototype  
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9 388 clone. Within E2 ORF, no variation was observed in three prototypic HPV 11 genomic variants  
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11 389 studied. In contrast, among seven non-prototypic HPV 11 genomic variants, one contained a  
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13 390 specific amino acid substitution N247T at nucleotide position 3642 and all non-prototypic  
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15 391 variants harbored a specific amino acid substitution K308R at nucleotide position 3645. In E4  
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17 392 ORF, four nucleotide mutations were detected and all resulted in amino acid exchanges. Amino  
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19 393 acid substitutions G61E and S78L detected at nucleotide positions 3436 and 3487 were  
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21 394 characteristic features of all seven non-prototypic HPV 11 genomic variants, while substitutions  
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23 395 Q46R and T70P detected at positions 3391 and 3462 constituted additional characteristics of  
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25 396 five and one non-prototypic HPV 11 genomic variants, respectively. Within L2 ORF, a 9 bp  
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27 397 insertion at position 5405 in the HPV 11-2 genomic variant (Fig. 3), which seemed to be a  
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29 398 duplication of an adjacent 9 bp segment (nt 5397-5405), resulted in three additional amino acids  
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31 399 (AAE). The 9 bp duplication was “in frame” in all respects in the DNA, so the protein has part of  
32  
33 400 its sequence repeated. Otherwise, no novel amino acid substitutions were found in L2 protein.  
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35 401 The biological and clinical significance of several novel amino acid changes detected in the E1,  
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37 402 E2, E4 and L2 genomic regions during full-length sequence comparison of 12 HPV 11 isolates  
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39 403 needs to be further investigated.

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41 404 The results of this study allowed for the first time phylogenetic comparison of full-length HPV 11  
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43 405 genomes. As shown in Fig. 4, full-length genome comparison of all HPV 11 isolates for which a  
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45 406 full-length sequence is available at present: 10 isolates from this study, prototype HPV 11 isolate  
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47 407 and isolate LZod45-11 (Wu et al., 2009), confirmed the existence of two major HPV 11 genomic  
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49 408 variant lineages, as suspected initially from the comparison of pooled L1, LCR, E6, and E5a  
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51 409 sequence data of all 63 HPV 11 isolates included in the study (Fig. 1). The obtained  
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53 410 phylogenetic trees were dichotomic, one branch representing the prototype HPV 11 and related  
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3 411 genomic variants and the other representing a group of HPV 11 genomic variants more distantly  
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5 412 related to the prototypic group. Considering that 10 phylogenetically evaluated HPV 11 genomic  
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7 413 variants represent more than 58% of HPV 11 isolates analyzed in this study, the observed  
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9 414 phenomenon can probably be extrapolated to the remainder of the isolates included in the study.  
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11 415 However, when extrapolating this observation further, one should keep in mind the sample bias  
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13 416 as most the important limitation of the present study. Namely, all samples included in the study  
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15 417 originated from an ethnogeographically closed cohort and there was a significant gender and  
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17 418 specimen type imbalance.

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20 419 In summary, the first extensive work on the genomic diversity of HPV 11 showed moderate  
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22 420 prevaccination genomic diversity of this HPV genotype within the ethnogeographically closed  
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24 421 cohort investigated. HPV 11 was shown to be less polymorphic in comparison to the majority of  
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26 422 HPV genotypes studied to date, including the most closely related genotype HPV 6. A total of 23  
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28 423 different HPV 11 genomic variants, composed of 11 L1, 12 LCR, 6 E6, 4 E5a and 4 E5b  
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30 424 genomic variants were identified. Non-prototypic HPV 11 genomic variants were found in the  
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32 425 majority of samples included in the study. Several novel, potentially important mutations were  
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34 426 described. Within the HPV 11 L1 protein, no amino acid changes that might significantly affect  
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36 427 the immunogenicity of HPV 11 were identified. These results will be of value in future  
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38 428 epidemiological, evolutionary history, functional, molecular assay development and vaccination  
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40 429 studies.  
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