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1 **The landscape of persistent human DNA viruses in femoral bone**

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13

14 **Keywords:** DNA viruses, femoral bone, NGS, human provenance, parvovirus B19 genotype 2

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18

19 **Abstract**

20 The imprints left by persistent DNA viruses in the tissues can testify to the changes driving virus  
21 evolution as well as provide clues on the provenance of modern and ancient humans. However, the  
22 history hidden in skeletal remains is practically unknown, as only parvovirus B19 and hepatitis B  
23 virus DNA have been detected in hard tissues so far. Here, we investigated the DNA prevalences of  
24 38 viruses in femoral bone of recently deceased individuals. To this end, we used quantitative PCRs  
25 and a custom viral targeted enrichment followed by next-generation sequencing. The data was  
26 analyzed with a tailor-made bioinformatics pipeline. Our findings revealed bone to be a much richer  
27 source of persistent DNA viruses than earlier perceived, discovering ten additional ones, including  
28 several members of the herpes- and polyomavirus families, as well as human papillomavirus 31 and  
29 torque teno virus. Remarkably, many of the viruses found have oncogenic potential and/or are likely  
30 to reactivate in the elderly and immunosuppressed individuals. Thus, their persistence warrants  
31 careful evaluation of their clinical significance and impact on bone biology. Our findings open new  
32 frontiers for the study of virus evolution from ancient relics as well as provide new tools for the  
33 investigation of human skeletal remains in forensic and archeological contexts.

## 34 **Introduction**

35 DNA viruses commonly establish persisting infections in humans, remaining thus, their genetic  
36 material imprinted in the tissues throughout life. These DNAs exhibit phylogeographies resembling  
37 those of humans[1], pointing to shared evolutionary and dispersal paths that can, together, add to  
38 the understanding of human population history and migrations[2–8]. In addition, the specific  
39 geographical distributions of DNA viruses, in both global[2,9] and local[10–12] scales, may provide  
40 new insights into the origins of unidentified individuals.

41 In this regard, the most widely studied virus is JC polyomavirus (JCPyV), a highly prevalent pathogen  
42 with three main phylogenetic clusters spread across Africa, Asia, and Europe. JCPyV's genotype-  
43 specific global spread has been proposed as an indicator of the provenances of both modern[12]  
44 and ancient humans[13–15]. Although its timescale of evolution has been debated[16–18], recent  
45 work by Forni et al.,[8] based on ~1100 worldwide strains, supports co-dispersal of this virus with  
46 major human migratory routes as well as its co-divergence with human mitochondrial and nuclear  
47 markers.

48 In addition to the spatial and temporal dimensions, viruses also display intriguing age-dependent  
49 distributions[19–21]. This is the case with genotype 2 of parvovirus B19, the traces of which went  
50 missing in Europe around 1970s. Thus, contemporary DNA findings of this virus variant in tissues  
51 are confined to elderly individuals or historic human remains[22].

52 Despite these encouraging data, the utility of the viral DNAs has not been fully addressed in forensic  
53 and anthropological settings. Reasons for this include the lack of a comprehensive picture of the  
54 overall distribution of viral DNAs in various organs, limiting the use of some human tissues in versatile  
55 scenarios, and most importantly, the fact that their prevalences in bones and teeth are almost entirely  
56 unknown.

57 Of the latter, we were the first to detect parvovirus B19 DNA in bones from soldiers of World War  
58 II[23], followed by findings of this same virus[24] and of hepatitis B[25,26] virus in archaeological  
59 remains dated from the Neolithic to medieval times. These singular discoveries confirm the presence  
60 and preservation of viral DNA in hard tissues and call for the investigation of the full landscape of  
61 viral DNAs that here persist.

62 In this study, we searched for 38 highly prevalent DNA viruses in the femoral bones of recently  
63 deceased individuals. To this end, we used in-house quantitative PCRs (qPCRs) and virus-targeted  
64 enrichment coupled with next-generation sequencing (NGS).

65 Our findings significantly expand the present knowledge on the virome of human bone, opening new  
66 frontiers for the study of virus evolution as well new tools for the investigation of forensic and  
67 anthropological cases.

## 68 **Materials and methods**

### 69 *Study subjects*

70 The study cohort consisted of 27 deceased individuals of Finnish origin, aged 36 to 85 years (mean  
71 67.6) with a male:female ratio of 19:8. The postmortem interval ranged from 4 to 30 days (mean 8).  
72 The manners of death were disease (n=19), occupational disease (n=1), injury (n=5), and suicide  
73 (n=2). In none of the cases the medical records revealed the use of immunosuppressants or pre-  
74 conditions related to infectious-diseases, except for one with a history of herpes-zoster a few weeks  
75 before death. The mode and cause of death are presented in Figure 2. The study protocol was  
76 reviewed by the Ethics Committee of Helsinki and Uusimaa Hospital District (approval  
77 164/13/03/00/2014).

### 78 *Specimen collection and preparation*

79 A 5-10 cm piece of the femoral diaphysis was collected and cut longitudinally with an oscillating tip  
80 saw. The bone was cleaned using a toothbrush and washed sequentially in distilled water, 0.1 %  
81 sodium hypochlorite, and 96.1 w/w ethanol. The bones were let to dry for 5 to 7 days at room  
82 temperature under flow in a laminar hood. From each individual, the external and internal surfaces  
83 of femoral bone were sampled using a dentist drill (Schick Qube) except for three cases, in which  
84 the bone was cryomilled (Spex 6775 Freezer/mill; Spex). The sample preparations and drillings were  
85 performed in enclosed dedicated facilities, using full-body protection suits.

### 86 *DNA extraction*

87 Approximately 0.1-0.4g of bone powder were lysed and extracted as specified before[23] and eluted  
88 in a final volume of 50-100 µl. The DNA extractions were performed in enclosed facilities, dedicated  
89 to the extraction of human DNA. No viral work is performed in these rooms.

90 The total DNA was quantitated with Quant-iT™ PicoGreen™ dsDNA Assay Kit (Thermo Fisher  
91 Scientific).

92 The quality of the total DNA was evaluated with the Genomic DNA Reagent Kit in a LabChip GX  
93 Instrument (Perkin Elmer).

### 94 *Quantitative PCRs*

95 The quantifications of parvovirus B19, Merkel cell, JC and BK polyomaviruses, and nine human  
96 herpesviruses as well as of the human single-copy gene RNase P were performed with in-house  
97 quantitative PCRs (qPCRs) as described[27–30] [Pyöriä *et al.* in press]. The quantification of  
98 hepatitis B virus and human papillomavirus type 31 DNAs were performed with commercial kits  
99 (Hepatitis B Virus PCR Kit, GeneProof; Genesig Human papillomavirus 31 Standard kit,  
100 PrimerDesign) according to the manufacturer instructions.

101 For the quantification of torque teno virus, a qPCR was optimized[31,32] to amplify and detect the  
102 conserved untranslated region (UTR) of the virus using the following primers: AMTS fwd (5'-  
103 GTGCCGNAGGTGAGTTTA-'3), AMTAS rev (5'-AGCCCGGCCAGTCC-'3), AMTASgr4 rev (5'-  
104 AGCCCGGCCAGACC-'3) and AMTPTU probe (5'-FAM-TCAAGGGGCAATTCGGGCT-BHQ1-'3).  
105 The qPCR reaction consisted of 1x Maxima probe qPCR Master mix (Thermo Fisher Scientific) with  
106 0.03 µM of ROX passive reference dye, 0.5 µM of each of the primers, 0.4 µM of the probe, 5 µl of  
107 the template, and nuclease-free water to a final volume of 25 µl. After initial denaturation at +95°C  
108 for 10 min, the qPCR cycles were +95 °C for 15 s and +55 °C for 1 min for 45 cycles.

109 The qPCR amplicons were 63 to 154 nucleotides in length.

110 Plasmid dilution series were used in all the qPCR runs as positive controls and to create standard  
111 curves for quantification. The plasmids of parvovirus B19, human herpesvirus 1-8, Merkel cell, JC  
112 and BK polyomaviruses, and RNaseP are described elsewhere[33][Pyöriä *et al.* in press]. For torque  
113 teno virus, a plasmid, named 10B, containing 1184 nucleotides of the virus was cloned from a healthy  
114 blood donor's plasma (GenBank MT448658).

115 The virus amplifications were completed with AriaMx Real-Time PCR System except for those of  
116 torque teno virus, RNaseP, and human papillomavirus 31 that were analyzed with Stratagene  
117 Mx3005P qPCR System (both Agilent).

118 The qPCR mixes, sample handling, plasmid dilutions, and amplifications, were performed each in  
119 completely separate rooms, following strict protocols and work-flows to prevent contamination.  
120 Negative controls (PCR-grade water) were included in every step starting from the DNA extraction.

#### 121 *PCR inhibition tests*

122 PCR efficiency due to carryover of inhibitors following DNA extraction was controlled using DNA  
123 extracts from bone together with or in parallel to a pre-quantified RNaseP plasmid[33].

124 To evaluate the performance of different polymerases and the quantification accuracy, four different  
125 commercial master mixes were tested. From this, Maxima probe qPCR master mix was selected  
126 and used throughout the study.

127 The impact of residual EDTA on the qPCR performance was examined by testing varying EDTA  
128 concentrations in the lysis buffer (0.5 to 500 nM) and by adding excess EDTA (0.05 to 50 nM) to the  
129 qPCR reaction. In addition, pre-quantified plasmid dilutions were extracted following the bone  
130 extraction protocol (i.e. lysis buffer containing 500 nM EDTA).

131 The impact of residual Ca<sup>2+</sup>-ions from bone in the extracts was investigated by addition of MgCl<sub>2</sub> to  
132 the qPCR reactions (in final concentrations of 4 to 7 mM).

133

134 *Library preparation, viral enrichment, and sequencing*

135 The sequencing libraries were prepared on 10 to 1000 ng of total DNA using the KAPA HyperPlus  
136 library preparation kit (Roche), following the manufacturer protocol with two modifications: 1)  
137 mechanical fragmentation with a Covaris E220 of the DNA with target fragments of 200 nt and 2) the  
138 use of xGen Dual Index UMI Adapters (Integrated DNA Technologies).

139 After sonication, the fragment length distributions were analyzed with the DNA High Sensitivity  
140 Reagent Kit in a LabChip GX Instrument (Perkin Elmer).

141 The viral enrichment was performed using two consecutive rounds of hybridization on individual  
142 samples following recommendations for low input DNA (MyBaits v4 kit; Arbor Biosciences). For each  
143 library, 200 ng per round of biotinylated RNA-baits were used. The baits were 100 nt in length and  
144 designed with 2X tiling (Supplementary Table 1 for a list of viruses). xGen Universal Blockers-TS  
145 Mix (Integrated DNA Technologies) were used to block unspecific binding to the adapters during  
146 hybridization.

147 During library preparation and viral enrichment, the libraries were amplified 3x13-25 cycles. The  
148 clean-up steps were performed with either KAPA Pure Beads (Roche) or MinElute PCR Purification  
149 Kit (Qiagen).

150 The enriched libraries were quantified with KAPA Library Quantification Kit (Roche) using Stratagene  
151 3005P qPCR System (Agilent) and subsequently pooled for sequencing on NovaSeq 6000 (SP  
152 PE151 reagent kit; Illumina).

153 *NGS data analysis*

154 The viral genomic sequences were reconstructed after removal of PCR duplicates, using a  
155 customized bioinformatics pipeline (TRACESPipe; [Pratas *et al.* in revision; available for download  
156 at <https://github.com/viromelab/tracespipe>).

157 The consensus, as well as single sequences (when in low coverage), were confirmed by BLAST  
158 (NCBI). The highest similarity was used to classify the virus genotype. For parvovirus B19, the  
159 sequences covering >70 % of the viral genome (n=7) were aligned with previously published full or  
160 near-full length sequences in EMBL-EBI Clustal Omega and analyzed with Bioedit v.7.2.5 (Ibis  
161 Biosciences).

162 For the following viruses, the consensus sequences will be available in GenBank with respective  
163 accession numbers: parvovirus B19 (7 sequences; MT410184-MT410190); human papillomavirus  
164 type 31 (MT410191); hepatitis B virus (MT410192); Merkel cell polyomavirus (MT410193).

165

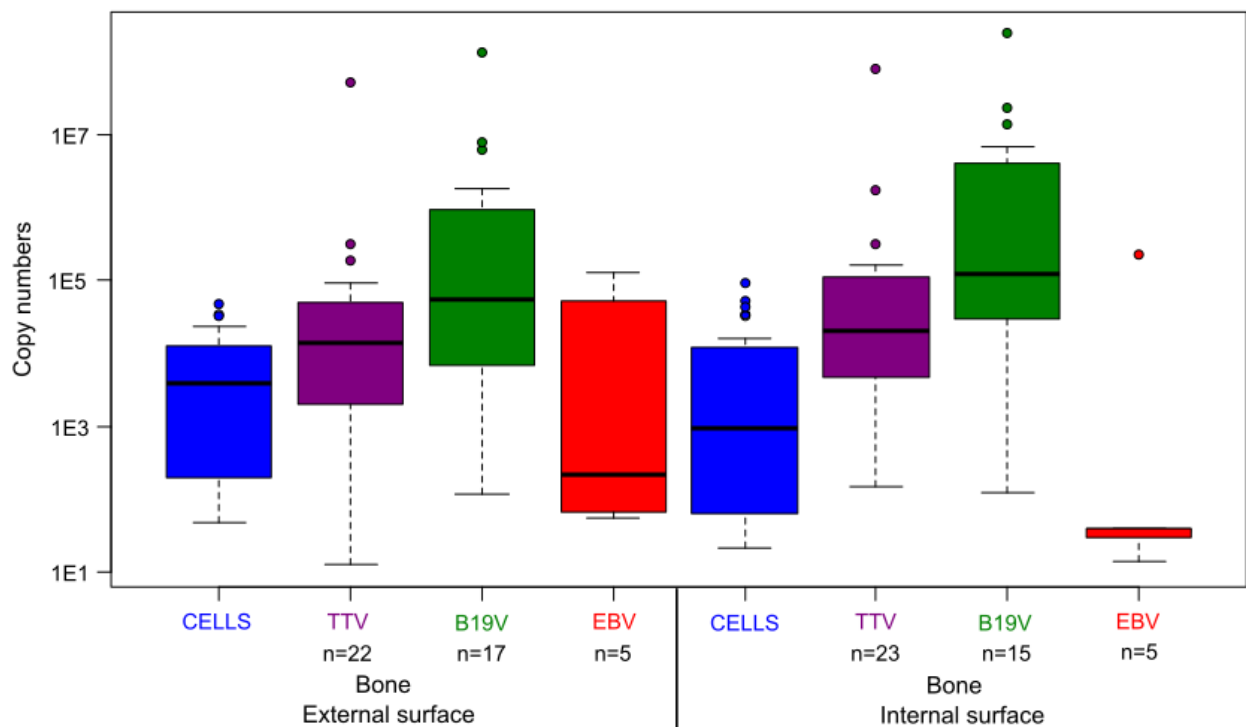
166 *Statistical analysis*

167 The differences in viral or cell copy numbers in the external and internal surfaces of femoral bone  
168 were calculated with Student's t-test in RStudio (version 1.0.153).

169 **Results**

170 *Viral DNA prevalences in the external and internal surfaces of the femoral bone.*

171 To determine the most optimal site for sampling, we first investigated the differences in prevalence  
172 and quantity of persisting viral DNAs in the external and internal surfaces of 27 femoral bones (Fig  
173 1). To this end, we examined three ubiquitous viruses (torque teno, parvovirus B19, and Epstein-  
174 Barr virus) known to infect >70% of the global population and to persist in several soft tissues in the  
175 body. To control for bias by uneven amounts of DNA in the extracts, we performed in parallel a qPCR  
176 for the human single-copy gene RNase P. We found no significant differences in the viral DNA  
177 prevalences ( $p > 0.1$ ) nor copy numbers ( $p$  values  $> 0.5$ ) between these two surfaces. Hence,  
178 subsequent analyses were performed only on samples taken from the external surface.



179

180 **Fig 1. Viral loads in the external and internal surfaces of the femoral bone.** The numbers of cells as well  
181 as the DNA copies of torque teno virus (TTV), parvovirus B19 (B19V), and Epstein-Barr virus (EBV) from the  
182 external and internal surfaces of femoral bone were determined. No significant differences between these two  
183 locations were observed ( $p > 0.5$ ). The cell counts are expressed per 1  $\mu$ l of DNA extract and the viral DNA  
184 copy numbers per 1E6 cells.

185 *Viruses are highly prevalent in human femoral bone*

186 We then investigated the prevalences of altogether 38 persistent virus genomes using targeted  
187 enrichment and confirmatory qPCRs. Overall, the 27 study subjects harbored on average 2.6 virus-

188 types in their femoral bones, with a maximum of seven in one individual. Altogether, we detected 12  
 189 different virus-types in 92.6 % of the bones, with only two individuals (> 60 years of age) being  
 190 completely negative for all viruses tested. The viral findings are presented in Table 1 and Figure 2.

191 The viruses most prevalent in bone were torque teno virus and parvovirus B19 with genoprevalences  
 192 of 81.5% and 74.1%, respectively (Table 1). The third most prevalent was Merkel cell polyomavirus  
 193 at 33.3%, followed by Epstein-Barr virus (25.9%), human papillomavirus (22.2%), human  
 194 herpesvirus 7 (18.5%) and JC polyomavirus (14.8%). Other viral sequences detected were of herpes  
 195 simplex 1, varicella-zoster, cytomegalovirus, human herpesvirus 6B, and hepatitis B virus.

196 The median viral copy numbers per one million cells were 1.9E4 for torque teno virus, 2.4E3 for  
 197 parvovirus B19, 8.7E3 for Merkel cell polyomavirus, and 2.1E1 for Epstein-Barr virus (Table 1).

198 The most common co-occurrences were of parvovirus B19 and torque teno virus, found in 70.3% of  
 199 the samples. Interestingly, we detected in the bone of one individual three cancer-associated viruses:  
 200 hepatitis B virus, human papillomavirus type 31, and Merkel cell polyomavirus.

201 **Table 1. Viral DNA prevalence in human femoral bone**

Family	Virus	Genome size (kb)	Geno-prevalence	NGS+ cases	qPCR+ cases	Breadth coverage (range or value of single sample)	Viral load (copies/million cells)
<b>Parvoviridae</b>	B19V	5.6	74.1 %	17	16	4.5-100.0 %	2.4E3
<b>Herpesviridae</b>	HSV1	152	3.7 %	1	1	3.4 %	4.9E1
	VZV	125	3.7 %	1	1	13.6 %	2.0E0
	EBV	170	25.9 %	6	4	1.0-3.3 %	2.1E1
	CMV	236	3.7 %	0	1	-	9.8E1
	HHV6B	162	11.1 %	3	0	2.0-3.1 %	-
	HHV7	150	18.5 %	5	0	4.8-12.0 %	-
<b>Polyomaviridae</b>	JCPyV	5.1	14.8 %	3	2	3.7-14.8 %	2.7E1
	MCPyV	5.4	33.3 %	9	4	1.4-58.6 %	8.7E3
<b>Papillomaviridae</b>	HPV	8	22.2 %	6	1*	1.8-89.6 %	8.7E3
<b>Hepadnaviridae</b>	HBV	3.2	7.4 %	2	1	15.6-44.5 %	1.7E3
<b>Anelloviridae</b>	TTV	3.8	81.5 %	9	22	2.1-56.3 %	1.9E4

202 \* Only HPV type 31 qPCR was performed. B19V: parvovirus B19; HSV1: herpes simplex virus-1; VZV: varicella-zoster virus; EBV:  
 203 Epstein-Barr virus; CMV: cytomegalovirus; HHV6B: human herpesvirus 6B; HHV7: human herpesvirus 7; JCPyV: JC polyomavirus;  
 204 MCPyV: Merkel cell polyomavirus; HPV: human papillomavirus; HBV: hepatitis B virus; TTV: torque teno virus

205 We verified the accuracy of the qPCR results by examining the patterns of DNA fragmentation in the  
 206 extracts and by evaluating the impact of potential inhibitors carried over during lysis and extraction.  
 207 The quality of the total DNA in each sample was analyzed with a LabChip GX Instrument, which  
 208 revealed 100 to 500 nt fragments in addition to intact genomic DNA ( $\leq 40$  kb; Fig 3). We found no  
 209 PCR inhibition accountable to excess EDTA or  $Ca^{2+}$  in the extracts.

210

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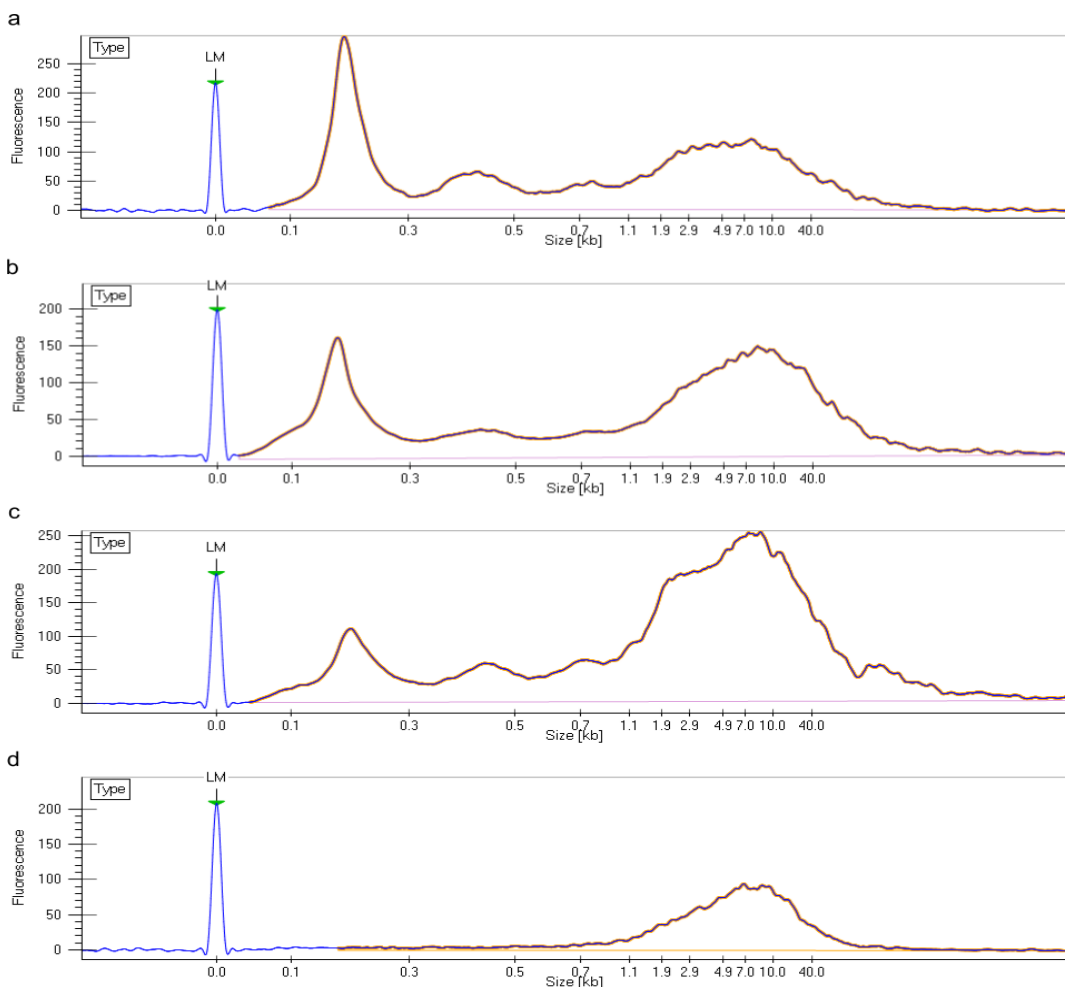
212

213

	cases																											
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	
COD*	F10	I35	C34	F71	W19	???	I25	W19	W19	F10	I25	T36	I11	I25	I25	I35	F50	I25	W79	G30	I25	F10	C32	X74	I25	J84	I25	
MOD^	1	1	2	1	3	5?	1	3	3	1	1	3	1	1	1	1	1	1	3	1	1	1	1	5	1	1	1	
B19V	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green
HSV1	White	White	White	White	White	White	White	White	White	White	White	White	White	White	White	White	White	White	White	White	White	White	White	White	White	White	White	White
VZV	White	White	White	White	White	White	White	White	White	White	White	White	White	White	White	White	White	White	White	White	White	White	White	White	White	White	White	White
EBV	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue
CMV	White	White	White	White	White	White	White	White	White	White	White	White	White	White	White	White	White	White	White	White	White	White	White	White	White	White	White	White
HHV6B	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue
HHV7	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue
JCPyV	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue
MCPyV	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue
HPV	White	White	White	White	White	White	White	White	White	White	White	White	White	White	White	White	White	White	White	White	White	White	White	White	White	White	White	White
HBV	White	White	White	White	White	White	White	White	White	White	White	White	White	White	White	White	White	White	White	White	White	White	White	White	White	White	White	White
TTV	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green

214 **Fig 2. Viral DNA occurrence by NGS and qPCR in bone per study subject.** The viral findings are  
 215 presented as green (NGS+, qPCR+), yellow (NGS+, qPCR-), orange (NGS-, qPCR+), and white  
 216 (NGS-, qPCR-). \*The cause of death (COD) is given according to the WHO ICD10 classification.  
 217 ^The manner of death (MOD) is presented as 1=disease, 2=occupational disease, 3=injury,  
 218 5=suicide. The results are presented from left to right according to the highest and lowest virus  
 219 prevalences in bone. B19V= parvovirus B19; HSV1=herpes simplex virus-1; VZV= varicella-zoster  
 220 virus; EBV= Epstein-Barr virus; CMV= cytomegalovirus; HHV6B= human herpesvirus 6B; HHV7=  
 221 human herpesvirus 7; JCPyV= JC polyomavirus; MCPyV= Merkel cell polyomavirus; HPV= human  
 222 papillomavirus; HBV= hepatitis B virus; TTV= torque teno virus.

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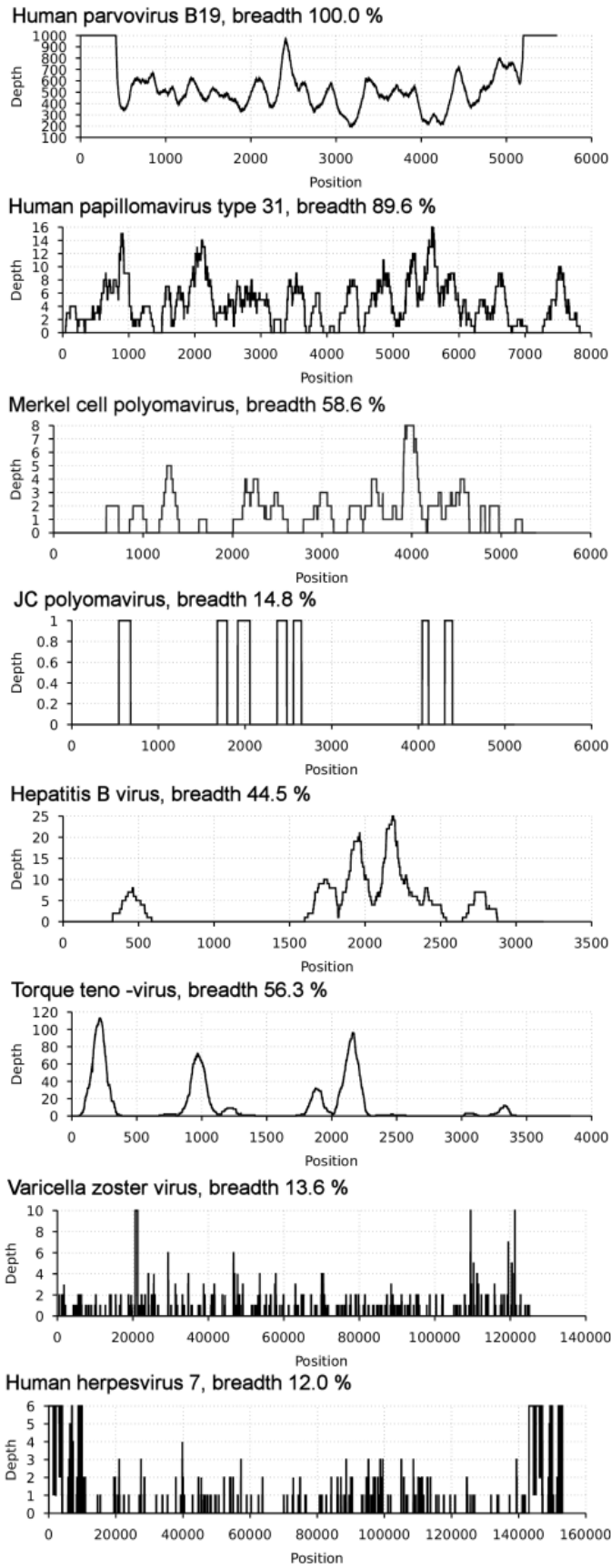


224 **Fig 3. DNA fragment analysis of total DNA.** Fragment length distribution of patterns of genomic  
 225 DNA for four representative samples as established with LabChip GX.

226 *Viral capture and sequence analysis*

227 As established from the qPCR results, the persisting viral DNA quantities were far below 1% of total  
228 DNA present in a sample. Thus, to enrich this fraction, we performed in-solution capture with  
229 biotinylated RNA oligonucleotides prior to sequencing in Novaseq 6000. Subsequently, we analyzed  
230 the NGS data with a custom pipeline, TRACESPipe (Pratas *et al.* in revision), which reconstructs the  
231 viral sequences using both reference-based alignment and *de-novo* assembly.

232 We reconstructed a total of 15 viral genomic sequences, with a minimum of 15% breadth coverage.  
233 The highest qualities were attained for parvovirus B19 (n=7; average breadth coverage 86.1%),  
234 human papillomavirus (n=1; breadth coverage 89.6%), Merkel cell polyomavirus (n=1; breath  
235 coverage 58.6%), JC polyomavirus (n=1; breadth coverage 14.8%), hepatitis B virus (n=2; breadth  
236 coverages 44.5% and 15.6%), and torque teno virus (n=3; breadth coverages 56.3%, 30.6% and  
237 24.9%). The breadth and depth coverages of representative viruses are presented in Figure 4.



238

239 **Fig 4. Coverage profiles of reconstructed viral DNA sequences.** NGS coverage profiles (breadth and  
 240 depth) of representative viruses.

241 By NCBI BLAST, we found close relation of the reconstructed genomes to previously published  
242 sequences. We recovered seven full/near-full B19V genomic sequences, three of which were of  
243 genotype 1 (95.16% to 99.61% similarity to AY504945.1) and four of genotype 2 (90.1% to 98.6%,  
244 similarity to AB550331.1). The latter genotype represents an extinct form of this virus and was found  
245 in the present study exclusively in individuals older than 68 years. Of this genotype, only a single  
246 full-length sequence has ever been published (AB550331.1). We found that this genotype's hairpins  
247 present similar flip and flop configurations to those of genotype 1.

248 The human papillomavirus sequence showed 99.1% similarity to type 31 (KU298889.1); Merkel cell  
249 polyomavirus 98.7% to the European/Caucasian type (KF266963.1) and JC polyomavirus 100.0%  
250 to genotype 1 (MF662198.1). The two hepatitis B virus sequences matched 99.4% and 99.6% to  
251 genotypes D (JX898691.1) and A (MN507849.1), respectively; and the torque teno virus sequences  
252 showed 90.2%, 99.0% and 96.2% similarity to strains AF122920.1, KT163880.1 and FR751497.1,  
253 respectively.

254 Moreover, we confirmed by BLAST unique sequences (mean length 100 nt) mapping to the following  
255 viruses: herpes simplex 1 (1 case, 28 reads), varicella-zoster (1 case, 362 reads), Epstein Barr (6  
256 cases, altogether 270 reads), human herpesvirus 6B (3 cases, total of 66 reads), human herpesvirus  
257 7 (5 cases, total of 274 reads), JC polyomavirus (3 additional cases, total of 13 reads), Merkel cell  
258 polyomavirus (8 additional cases, total of 15 reads), human papillomavirus (5 additional cases, total  
259 of 14 reads), torque teno virus (6 additional cases, total of 26 reads), and parvovirus B19 (10  
260 additional cases, total of 673 reads).

## 261 **Discussion**

262 The analysis of viral DNAs shows great potential as complementary markers for human identification  
263 as well as for estimation of provenance and migration. However, one limitation of their use in these  
264 contexts is insufficient knowledge of the landscape of viruses persisting in the host, in particular in  
265 human bone, with only parvovirus B19[23,24] and hepatitis B[25] virus having been detected in this  
266 tissue so far.

267 To this end, we systematically explored the prevalences of 38 ubiquitous viruses in human femoral  
268 bone. We discovered an unprecedented number of viral DNAs, detecting up to seven per individual.  
269 Besides the already known, we report here on ten new viruses including several members of the  
270 herpesvirus family (herpes simplex-1, varicella-zoster, Epstein-Barr, cytomegalovirus, human  
271 herpesviruses 6B and 7), JC- and Merkel cell polyomaviruses, human papillomavirus 31, and torque  
272 teno virus. Intriguingly, a common feature shared by these viruses (except for HHV-6B) is their  
273 persistence in soft tissues in episomal form[34]. Although the methods used in this study prevent us  
274 from confirming whether this is also the case in bone, the low copy numbers detected are indicative  
275 of a quiescent infection. Moreover, some of them (e.g. herpes-, polyoma-, and papillomaviruses) are

276 known to establish latency as densely packed nucleosomes, as mechanism to regulate gene  
277 expression[35,36]. Thus, the circular form and the tight histone packaging may confer DNA viruses  
278 superior preservation in relation to the host DNA (nuclear and mitochondrial)[37–39]. In this regard,  
279 the analysis of viral DNA could be of utmost value for forensic investigations or studies of ancient  
280 human remains dealing with highly compromised samples.

281 One challenging factor in the study of the human virome continues to be its extremely low proportion  
282 in relation to other sources of DNA. With this in mind, we carried out our screening using two  
283 approaches, qPCR and NGS. We found that the viral DNAs were exceeded on average a 1000-fold  
284 by the nuclear DNA alone, in line with the loads reported for persisting viruses in soft tissues[40–42].  
285 By targeted enrichment and NGS we identified a higher number of viruses than by qPCR, an  
286 observation that can in part be explained by moderate DNA fragmentation in the samples. The only  
287 exception was torque teno virus (TTV), for which we found a significantly higher number of positive  
288 samples by qPCR. This circular ssDNA virus exhibits substantial heterogeneity[43], which was  
289 under-represented by the three reference sequences upon which our baits were designed. Hence,  
290 a better characterization of it could require a broader bait coverage of its five genogroups together  
291 with analysis at the amino acid level, as certain motifs are more likely to be shared by different TTVs.  
292 The qPCR on the other hand, targeted the conserved UTR region, to recognize a wide a repertoire  
293 of TT-viruses.

294 Unsurprisingly, larger genome coverages were recovered from the viruses with the highest copy  
295 numbers ( $\sim\log_3$  copies/million cells). However, many of the viral loads fell below this threshold,  
296 whereby, follow-up singleplex enrichments may be beneficial to increase the analytical resolution of  
297 the data. Importantly, even for the low coverage genomes, we confirmed the sequences retrieved to  
298 be virus-specific and hence, genuine findings of the femoral bone.

299 In all, we unveil that the human femoral bone is a much richer source of persistent DNA viruses than  
300 earlier known. We propose that a “Human Virome Panel” could be built as an efficient tool for  
301 assessment of human provenance in conjunction with the standard human DNA profiling. Indeed,  
302 the phylogeographical distributions of JC[13,14,44], BK[2], varicella-zoster[7], and Epstein Barr[6]  
303 viruses have been examined in this context and shown to add power to forensic cases and  
304 anthropological studies. Undoubtedly, larger cohorts, both global and local, are required to validate  
305 the benefits and boundaries of such panel in multiple taphonomic conditions. This work could also  
306 help to identify patterns in the age distributions of certain viruses and their genotypes[19,20], to  
307 support biometric estimations.

308 Moreover, our data raise intriguing questions on the clinical significance of the long-term presence  
309 of these viruses – or their genomes – in the human skeleton. Indeed, many of the viruses we found  
310 can reactivate, and several have oncogenic potential. Among the latter, we detected Merkel cell

311 polyomavirus and human papillomavirus 31, two oncoviruses which, remarkably, are  
312 mucocutaneous. Certainly, these unexpected findings warrant further studies on the transcriptional,  
313 translational and reactivation potential of these pathogens as bone residents, particularly among the  
314 elderly and immune suppressed.

### 315 **Conclusions**

316 Our work substantially expands the current knowledge on the spectrum of DNA viruses persisting in  
317 human bone and opens new perspectives on their applicability in the investigation of human skeletal  
318 remains. It also supports the search for viruses from ancient relics, which can foreseeably remodel  
319 our understanding of virus evolution.

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330

### 331 **Conflicts of Interest**

332 The authors declare no conflict of interest.

### 333 **Contributions**

334 M.T., K.H., M.F.P and A.S designed the study. M.T. executed the experiments. MT, D.P. and  
335 M.F.P analyzed the data, E.V. and M.S.V contributed with a plasmid and qPCR optimization, M.T.,  
336 M.F.P. and A.S. drafted the manuscript. All authors commented, edited and approved the final  
337 version of the manuscript.

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