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Running title: New KSHV variant identified in MSM

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► **To cite this version:**

Aude Jary, Valentin Leducq, Nathalie Désiré, Héloïse Petit, Romain Palich, et al.. New Kaposi's sarcoma-associated herpesvirus variant in men who have sex with men 3 associated with severe pathologies Running title: New KSHV variant identified in MSM. *Journal of Infectious Diseases*, 2020, 222 (8), pp.1320-1328. 10.1093/infdis/jiaa180 . hal-02996334

HAL Id: hal-02996334

<https://hal.sorbonne-universite.fr/hal-02996334v1>

Submitted on 9 Nov 2020

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1 **Category:** Major article

2

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8 Aude JARY¹, Valentin LEDUCQ¹, Nathalie DESIRE¹, Héloïse PETIT¹, Romain PALICH²,
9 Véronique JOLY³, Ana CANESTRI⁴, Adélie GOTHLAND¹, Sidonie LAMBERT-NICLOT⁵,
10 Laure SURGERS⁶, Corinne AMIEL⁷, Diane DESCAMPS⁸, Jean-Philippe SPANO⁹, Christine
11 KATLAMA², Vincent CALVEZ¹, Anne-Geneviève MARCELIN¹

12

13 ¹Sorbonne Université, INSERM, Institut Pierre Louis d'Epidémiologie et de Santé Publique
14 (iPLESP), AP-HP, Pitié Salpêtrière Hospital, Department of Virology, F-75013 Paris, France

15 ²Sorbonne Université, INSERM, Institut Pierre Louis d'Epidémiologie et de Santé Publique
16 (iPLESP), AP-HP, Pitié Salpêtrière Hospital, Department of Infectious Diseases, F-75013
17 Paris, France

18 ³IAME, UMR 1137, INSERM, Université Paris Diderot, Sorbonne Paris Cité, AP-HP,
19 Service de Maladies Infectieuses et Tropicales, Hôpital Bichat, AP-HP, Paris, France

20 ⁴Service de Maladies Infectieuses et Tropicale, AP-HP Hôpital Tenon, Paris, France

21 ⁵Sorbonne Université, INSERM, Institut Pierre Louis d'Epidémiologie et de Santé Publique
22 (iPLESP), AP-HP, Hôpital Saint Antoine, Service de Virologie, Paris, France

23 ⁶Sorbonne Université, INSERM, Centre d'Immunologie et des Maladies Infectieuses (CIMI),
24 AP-HP, Hôpital Saint Antoine, Service de Maladies Infectieuses et Tropicales, Paris, France

25 ⁷Service de Virologie, AP-HP Hôpital Tenon, Paris, France

26 ⁸IAME, UMR 1137, INSERM, Université Paris Diderot, Sorbonne Paris Cité, AP-HP,
27 Service de Virologie, Hôpital Bichat, AP-HP, Paris, France

28 ⁹Sorbonne Université, INSERM, Institut Pierre Louis d'Epidémiologie et de Santé Publique
29 (iPLESP), AP-HP, Hôpital Pitié Salpêtrière, Service d'Oncologie Médicale, Paris, France

30

31

32 **Summary**

33 We identified a new Kaposi sarcoma-associated herpesvirus F variant in 5 Caucasian men
34 who have sex with men. Careful screening may be required in this population, given the
35 severe clinical presentation of associated diseases in the context of immunosuppression.

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51 **Abstract** (word count=200)

52 **Background:** Kaposi's sarcoma-associated herpesvirus (KSHV) subtype depends mostly on
53 patient origin. This study aimed to assess KSHV-diversity in a population of MSM living in
54 France.

55 **Methods:** We included 264 patients; (i) 65 MSM: 57 HIV-infected with Kaposi's sarcoma
56 (KS), multicentric Castleman disease (MCD) or primary effusion lymphoma (PEL), and 8
57 HIV-uninfected on HIV-pre-exposure prophylaxis (PrEP) to perform KSHV-typing by ORF-
58 K1 Sanger and KSHV-whole-genome sequencing, (ii) 199 other patients for real-time PCR
59 screening for the new variant.

60 **Results:** We found that 51% of KSHV-strains were subtype C (85% C3) and 33% were
61 subtype A. Four patients with severe KSHV-disease (2 visceral KS, 1 MCD, 1 PEL) and one
62 asymptomatic PrEP user had a new variant resembling the Ugandan subtype F, but with
63 different ORF-K1 and KSHV-whole-genome sequences and a different epidemiological
64 context (MSM *versus* African population). Its prevalence was 4.5% in Caucasian MSM and
65 absent in other epidemiological groups.

66 **Conclusions:** Subtype C predominated among MSM living in France. The new F-variant was
67 identified in Caucasian MSM and associated with severe KSHV-disease, suggesting that
68 subtype F could be split into F1 and F2 variants. Careful screening for this variant may be
69 required in MSM given the severe clinical presentation of associated diseases.

70

71 **Keywords:** KSHV, MSM, new variant, ORF-K1, phylogenetic analysis, whole-genome
72 sequencing

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76 Word count = 2671

77 INTRODUCTION

78 Kaposi's sarcoma-associated herpesvirus (KSHV) is the recognized etiologic agent of
79 all epidemiological forms of Kaposi's sarcoma (KS), including classic, endemic, post-
80 transplant and epidemic (HIV-associated) forms. This virus is also involved in the
81 development of two lymphoid malignancies: primary effusion lymphoma (PEL) and
82 multicentric Castleman disease (MCM), mostly in immunocompromised patients.

83 The K1 open reading frame (ORF-K1) encodes a transmembrane glycoprotein with an amino-
84 acid sequence varying by 20% to 44% between KSHV subtypes and by about 10% within
85 subtypes (genotype variant) [1]. A molecular epidemiological analysis of ORF-K1 led to the
86 identification of seven KSHV subtypes (A, B, C, D, E, F and Z), the distribution of which
87 worldwide depends on patient origin. Subtypes A and C were found in Europe, North
88 America, the Middle East, the Mediterranean and Asia [2–4]; subtypes B and A5 were found
89 predominantly in sub-Saharan Africa [5]; subtype D were found on Pacific islands and in
90 Taiwan [6]; subtype E was found in Brazilian Indians [7,8], subtype F in individuals from
91 Uganda [9] and subtype Z was identified in a small cohort of Zambian children [10].

92 Among men who have sex with men (MSM), KSHV-seroprevalence is higher than in the
93 general population of Western Europe (<5%) [11]. A recent systemic review and meta-
94 analysis reported a pooled KSHV-seroprevalence in both HIV-infected and uninfected MSM
95 of 33% [12]. The C and A subtypes of KSHV are the most prevalent in European and Asian
96 MSM with KSHV-associated diseases [13–15], consistent with the prevalence of KSHV
97 subtypes in the general populations of these regions [1–3]. However, although the KSHV
98 subtype distribution depends mostly on the patient's region of origin, several studies have
99 suggested that it may also be affected by clinical presentation or progression, particularly for
100 different forms of KS. For example, the A5 variant has been associated with extensive disease

101 in epidemic KS form [5], the A subtype has been associated with rapidly evolving classic KS
102 form [16] and the A and B' subtypes have been shown to contrast with the C subtype by
103 occurring at extracutaneous sites in post-transplant KS form [17].

104 We conducted a retrospective study analyzing HIV-infected MSM living in France
105 with KS, MCD or PEL, to describe KSHV subtype diversity and potential associations with
106 the severity of clinical presentation. As it was clearly demonstrated that KSHV-prevalence
107 infection correlated with the number of MSM partners [18] and considering the fact that this
108 population could be exposed to KSHV-transmission by frequenting dense sexual network, we
109 also included HIV-seronegative MSM under HIV pre-exposure prophylaxis (PrEP) program
110 to compare KSHV strains.

111

112

113 **METHODS**

114 *Study population*

115 We studied 65 MSM: (i) 57 HIV-infected patients with KSHV-associated diseases diagnosed
116 between 2012 and 2017 in France and (ii) 8 MSM HIV-uninfected participants on HIV-PrEP
117 with positive KSHV-antibodies and displaying KSHV-DNA shedding in a buccal swab
118 sample. Demographic and medical data were collected, including age, country of origin, CD4
119 count, HIV-RNA viral load, KSHV-DNA viral load and clinical presentation of KSHV-
120 associated diseases. Severe clinical presentation was defined as visceral KS or lymphoid
121 malignancies.

122 We subsequently included 199 patients testing positive for KSHV-DNA since 2013,
123 diagnosed in our department, to estimate the prevalence of the new variant identified. For
124 each patient, demographic data were collected, including sex, age, sexual orientation, country
125 of origin and KSHV-DNA viral load.

126

127 ***KSHV typing***

128 For HIV-infected patients, we obtained a whole-blood sample at the time of KSHV disease
129 diagnosis. For PrEP-using MSM, we obtained a buccal swab sample during standard medical
130 follow-up. DNA was extracted from this samples and subjected to real-time PCR to amplify
131 both ORF-73 (encoding the latency-associated nuclear antigen, LANA) and the albumin gene,
132 as previously described [19].

133

134 • *Sanger sequencing of ORF-K1 or VR1*

135 A 679 bp fragment of ORF-K1 including the two hypervariable regions, VR1 (amino acids
136 (aa) 54 to 93) and VR2 (aa 191 to 228), was amplified by nested-PCR as previously described
137 [20]. If ORF-K1 amplification was unsuccessful, a second nested-PCR was performed to
138 amplify a 363 bp fragment including only VR1, as previously described [2]. Bidirectional
139 sequencing was performed with BigDye Terminator chemistry (Thermo Fisher Scientific®),
140 with analysis of the reaction products on an ABI sequencer.

141

142 • *KSHV whole-genome sequencing and assembly*

143 Sample libraries preparations and target enrichment were performed according to the *SeqCap*
144 *EZ HyperCap workflow* (Roche®). The DNA sample was fragmented mechanically and
145 specific adapters were added. DNA libraries were pooled and enriched in KSHV sequences
146 by two rounds of hybridization with 100 bp overlapping DNA probes designed in
147 conjunction with Roche® on the KSHV GK18 (AF148805.2) sequence (excluding repeat
148 regions) and on 28 ORF-K1 reference sequences available from the NCBI database (see
149 Supplementary Table 1), to give 5x coverage. Finally, next-generation sequencing was

150 performed with paired-end reads (Mid Output Kit v2, 2x75 bp) on the NextSeq500 Illumina®
151 system.

152 Reads were trimmed with Trimmomatic, using a quality (Q) threshold such that only bases
153 with $Q > 30$ were retained and reads of less than 50 bp were filtered out. Paired-end reads were
154 first mapped onto the KSHV reference sequence (GK18) with Bowtie 2.3.4.3. They were then
155 assembled *de novo* with Spades3.12.0 and Mira1.1.1 to generate two other sequences. Finally,
156 we used Mauve1.1.1 in Geneious11.1.4 to align the three sequences and establish the
157 consensus whole-genome KSHV sequence (KSHV-WG).

158

159 • *Phylogenetic analysis*

160 We performed a phylogenetic analysis of ORF-K1 amino-acid sequences and KSHV-WG
161 nucleotide sequences by the maximum likelihood method. Multiple sequence alignments were
162 generated with Mafft7 [21], and a phylogenetic analysis was then performed with PhyML3.0
163 [22] and 1000 bootstraps resampling. Pairwise genetic distances between ORF-K1 amino-acid
164 sequences were calculated with Mega7.0.14 [23], using the JTT model of substitutions and a
165 gamma distribution with 4 parameters.

166

167 ***Screening for the new F-variant by real-time PCR***

168 Whole-blood samples with positive KSHV-DNA viral load ($n=199$) were screened for the
169 new variant with a specific real-time PCR. We designed specific primers and probes with
170 Geneious 11.1.4, to amplify a fragment of about 120 bp in length encompassing the VR1
171 region and specific for the new KSHV variant (see Supplementary Table 2). The specificity of
172 that PCR was assessed by testing 25 different subtypes/genotype variants and the sensitivity at
173 25 copies/ 10^6 cells.

174

175 ***Statistical analysis***

176 Continuous variables were expressed as the median and interquartile range [IQR] and discrete
177 variables were expressed as numbers and percentages. GraphPad was used to perform non-
178 parametric tests, specifically Mann-Whitney U tests for quantitative data and Fisher's exact
179 test for qualitative data.

180

181 ***Ethics statement***

182 The study was carried out in accordance with the Declaration of Helsinki. It was a
183 retrospective non-interventional study with no addition to standard care procedures.
184 Reclassification of biological remnants into research material after completion of the ordered
185 virological tests was approved by the local interventional review board of Pitié-Salpêtrière
186 hospital. According to the French Public Health Code (CSP Article L.1121-1.1) such
187 protocols are exempted from individual informed consent.

188

189 **RESULTS**

190 ***Patients' characteristics***

191 In total, 41 of the HIV-infected men had been diagnosed with KS, 12 with MCD and 4 with
192 PEL. Most were Caucasian (50/57, 88%); 46/57(81%) originated from France, 6/57 (10%)
193 from the Mediterranean Basin, 4/57 (7%) from South America and 1/57 (2%) from Asia
194 (Table 1). KSHV-DNA viral load in whole blood was lower in patients with KS, than in those
195 with MCD or PEL ($p<0.0001$). Most of the PrEP users were also Caucasian (7/8, 88%); 4
196 (50%) originated from France, one from Switzerland, two from Eastern Europe and one from
197 South America. None of the PrEP users were infected with HIV or had KSHV-associated
198 diseases.

199

200 ***ORF-K1 phylogenetic analysis***

201 ORF-K1 amplification was successful for 34 of the 57 MSM with KSHV diseases (60%), and
202 related to the rate of KSHV-DNA viral load. For patients with all KSHV-related diseases
203 considered together, subtype C was the most prevalent [18/34 (53%)], followed by subtype A
204 [11/34 (32%)] and finally subtype B [1/34 (3%)]. For subtype C, 15/18 cases (83%) were
205 classified as the C3 variant. Subtype classification was possible for five of the eight PrEP
206 users (63%): the C3 variant in two cases and the A4 variant in two cases (Figure 1) (see
207 Supplementary Table 3). The genetic distance (GD) between subtypes B and A was 34%, that
208 between subtypes B and C was 39%, and the ORF-K1 amino-acid sequences of subtypes A
209 and C differed by 19%. Within subtypes, genotype variant differed from 9% (subtype A) and
210 from 13% (subtype C) at amino-acid level.

211 Interestingly, 5 KSHV strains (from P030, P035, P075, P076 and PrEP004) were closely
212 related to the F subtype described in an African population from Uganda (AAX55469_F) [9].
213 However, their amino-acid sequences differed from those of the F subtype by 11% and were
214 separated with a bootstrap confidence of 54% (Figure 1).

215 Overall, we found 3 clusters, corresponding to the C3 variant (17/39), the A4 variant (7/39)
216 and the “F subtype” (5/39). In most cases, the geographic origin of the MSM was consistent
217 with the subtype detected. Subtypes A and C were the most prevalent in patients originating
218 from France, the Mediterranean Basin and Asia (see Supplementary table 3).

219

220 ***KSHV subtype and clinical presentation***

221 The proportions of patients with KS and MCD did not differ significantly between subtypes:
222 26% of KS patients and 36% of MCD patients had subtype A viruses ($p=0.69$) and 63% of
223 KS and 55% of MCD patients had subtype C viruses ($p=0.71$), suggesting that the subtypes

224 involved in these two diseases may reflect the prevalence of the various subtypes in this
225 population.

226 Among patients with KS: (i) KSHV-DNA viral load tended to be higher for subtype A than
227 for subtype C ($p=0.051$), regardless of immunovirological status (Figure 2), (ii) the C3 variant
228 was more associated with purely cutaneous and/or oral-mucous involvement than the other
229 subtypes (odds ratio=11.6, 95% CI: 1.1-214.2, $p=0.023$), regardless of immunovirological
230 status ($CD4^+$ T-cell counts: C3 variant: $70/mm^3$ [21-325] vs. other subtypes: $82.5/mm^3$ [68-
231 213], $p=0.78$ - HIV-RNA viral load: C3 variant: $5.62 \log_{10}$ copies/ml [1.60-5.89] vs. other
232 subtypes: $5.38 \log_{10}$ copies/ml [4.71-5.65], $p=0.75$).

233

234 ***New KSHV F variant***

235 A new genotype variant closely resembling the F subtype was detected in five MSM, four of
236 whom had severe clinical presentation: two cases of visceral KS (P030 with cutaneous and
237 pulmonary involvement and P035 with cutaneous, pulmonary and ganglionic involvement),
238 one case of MCD associated with visceral KS (P075), and one case of PEL associated with
239 cutaneous KS (P076). The remaining individual was an asymptomatic PrEP user (PrEP004).
240 All these individuals were Caucasian (four from France and one from Peru) (see
241 Supplementary Table 4).

242 • *ORF-K1 Sanger sequencing*

243 Partial ORF-K1 sequences, 191 aa long (P030, P075, P076) and encompassing VR1 and VR2,
244 or 142 and 141 aa long (from P035 and PrEP004, respectively) and encompassing only VR1,
245 were generated; all sequences were strictly identical. The longest fragment differed by 18 aa
246 from the reference subtype F sequence from Uganda (AAX55469_F), and an insertion of 5 aa
247 was detected in VR2 (Figure 3). Otherwise, these sequences were closest ($GD=10^{-6}$) to the
248 KSHV sequence described in 2000 from a French HIV-positive MSM patient with PEL

249 (AAG01610_F) [15], from which they differed by only one amino acid, in position 202
250 (Figures 1 and 3).

251 We also compared these sequences with that for a subtype F KSHV strain from our database
252 that was obtained from a Congolese woman with MCD (P072). The ORF-K1 sequence from
253 this woman differed by 23 aa from our virus and by 20 aa (15 aa and the insertion described
254 above) from AAX55469_F. However, P072 and AAX55469_F clustered together in the
255 phylogenetic tree and this sequence was considered to correspond to an “African” F subtype
256 (Figures 1 and 3).

257 • *KSHV whole-genome sequencing*

258 For the formal identification of a new F variant, we performed KSHV-WG sequencing on the
259 four KSHV strains newly identified from patients with KSHV-related diseases, and compared
260 the sequences obtained with that for the P072 virus identified as belonging to the “African” F
261 subtype, because no KSHV-WG F subtype reference sequence was available in the NCBI
262 database. A consensus KSHV-WG sequence was obtained for all patients other than P035
263 (see Supplementary Table 4).

264 As in the phylogenetic analysis for ORF-K1, the phylogenetic tree generated for KSHV-WG
265 sequences presented a separation of subtype B from subtype A/C/F, with a bootstrap
266 confidence level of 100%. However, subtypes A and C were not clearly separated, suggesting
267 the probably involvement of other genes in KSHV variability, as well as recombination
268 events, as previously reported [24,25]. Phylogenetic analysis also confirmed that the two F
269 variants (Caucasian MSM *versus* African) differed and were separated with a bootstrap
270 confidence level of 61% (Figure 4). Although ORF-K1 amino-acid sequences were strictly
271 identical between P030, P075 and P076, KSHV-WG phylogenetic tree showed that new F
272 variant from KS patients differed from that in patients with lymphoid malignancies.

273 • *Prevalence of the new F variant*

274 Finally, we screened all available samples testing positive for KSHV-DNA since 2013
275 ($n=199$) with a new F variant-specific real-time PCR. The median KSHV-DNA viral load was
276 $1.92 [1.45-2.69] \log_{10}$ copies/ 10^6 cells. The patients tested had a median age of 54 [42-62.5]
277 years and 78% were men (156/199); 47% were Caucasian (73/156), and 77% (56/73) of these
278 Caucasians were MSM. None of the 199 samples tested was positive for the new F variant.
279 Overall, this new F variant was only described in Caucasian MSM patients with a prevalence
280 of 4.5% (5/113) in this population.

281

282

283 **DISCUSSION**

284 This study aimed to assess KSHV diversity in a population of MSM with and without
285 KSHV-associated disease living in France and to evaluate the possible correlation between
286 subtype and clinical presentation.

287 The global distribution of KSHV subtypes and variants in our MSM population was
288 consistent with the findings of previous studies conducted in France [15] and other countries
289 (Germany and Taiwan) [13,14], with KSHV subtypes C ($n=18$) and A ($n=11$) the most
290 prevalent.

291 We also found that the C3 variant was the most prevalent KSHV strain in a population of
292 MSM living in France, and that this variant was associated with a less severe clinical
293 presentation of the epidemic form of KS. In patients with epidemic KS, KSHV-DNA viral
294 load tended to be higher for subtype A than for subtype C. These results are consistent with
295 those of previous studies reporting a more aggressive clinical presentation in patients with
296 subtype A than in those with subtype C, for the classic and post-transplantation forms of KS
297 [16,17].

298 Although subtype F has been described only a few times in the literature [9,26,27], we
299 identified a new KSHV variant closely related to the first subtype F virus described in
300 Uganda. This new F variant was identified by Sanger sequencing and confirmed by whole-
301 genome sequencing on various samples from five patients. Based on the results of whole-
302 genome sequencing, we were able to classify this KSHV strain as a new F variant. Finally,
303 based on our results, we propose the subdivision of subtype F into two variants: F1 variant for
304 the KSHV described in Uganda and F2 variant for the KSHV identified in Caucasian MSM.
305 All the patients harboring this new F2 variant were Caucasian MSM living in Paris. The
306 screening of all our available samples since 2013 did not detect this new variant in other
307 patients, which suggests that it is present in a small, restrictive population. The overall
308 prevalence of the F2 variant was 4.5% (5/113) in the Caucasian MSM population, and this
309 variant was absent from other epidemiological groups. The patients harboring the new F2
310 variant included one immunocompetent PrEP user who was found to be merely a carrier, with
311 oral shedding but with no KSHV-related disease. The other four patients harboring the new
312 variant were immunocompromised (AIDS) and had severe forms of KSHV disease. Further
313 investigations are required to confirm these results and to determine whether specific subtypes
314 or viral determinants of virulence involved in KSHV tumor-associated processes can lead to
315 severe or persistent KSHV-related diseases, as recently reported for KS in HIV-infected
316 patients on effective antiretroviral therapies [28,29].

317

318 In conclusion, careful screening of the MSM population may be required for this new F2
319 variant, which is circulating in Caucasian MSM living in Paris, given the severe clinical
320 presentation of associated diseases in the context of immunosuppression.

321

322

323 **ACKNOWLEDGMENT**

324

325 **Funding.** This work was funded by the *Agence Nationale de Recherche sur le SIDA et les*
326 *Hépatites Virales* (ANRS) AC43 “sexually transmitted infections” working group.

327

328 **Conflicts of interest.** AJ, VL, ND, HP, RP, VJ, AC, AG, SLN, LS, CA, JPS, CK and VC
329 have nothing to disclose. DD reports personal fees from Gilead Sciences, personal fees from
330 MSD, personal fees from ViiV Healthcare, personal fees from Janssen-Cilag, outside the
331 submitted work. AGM reports grants and personal fees from VIIV Healthacare, grants and
332 personal fees from Gilead, grants and personal fees from MSD, personal fees from Janssen-
333 Cilag, outside the submitted work.

334

335 **Meeting presentation.** This work was presented as a poster discussion at the Conference on
336 Retroviruses and Opportunistic Infections (CROI), in March 2019 (Themed discussion:
337 KSHV virology and pathogenesis, poster no. 273).

338

339 **Corresponding author:** Aude Jary, 47-83 boulevard de l’hôpital, 75013 Paris,
340 +33142177406, aude.jary@aphp.fr

341

342 **Alternate corresponding author:** Anne-Geneviève Marcelin, [anne-](mailto:anne-genevieve.marcelin@aphp.fr)
343 [genevieve.marcelin@aphp.fr](mailto:anne-genevieve.marcelin@aphp.fr)

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452 **Table 1:** Epidemiological and medical characteristics of the 57 HIV-infected MSM with
 453 KSHV-associated diseases and the eight PrEP users

	HIV-infected			PrEP users
	KS	MCD	PEL	Positive KSHV-antibodies
No. of participants (%)	41/57 (63)	12/57 (19)	4/57 (6)	8/8 (100)
No. of MSM (%)	41/41 (100)	12/12 (100)	4/4 (100)	8/8 (100)
Median age [IQR], years	42 [35-53]	41 [37.75-53.75]	52 [46.75-53.75]	39 [35-42.5]
Country of origin, No. of participants (%)				
France	31/41 (76)	11/12 (92)	4/4 (100)	4/8 (50)
Western Europe (other than France)	0/41 (0)	0/12 (0)	0/4 (0)	1/8 (12.5)
Eastern Europe	0/41 (0)	0/12 (0)	0/4 (0)	2/8 (25)
Mediterranean Basin	5/41 (12)	1/12 (8)	0/4 (0)	0/8 (0)
South America	4/41 (10)	0/12 (0)	0/4 (0)	1/8 (12.5)
Asian	1/41 (2)	0/12 (0)	0/4 (0)	0/8 (0)
HIV infection				
No. (%)	41/41 (100)	12/12 (100)	4/4 (100)	0/8 (0)
Median CD4 ⁺ T-cell count [IQR], cells/mm ³	82.5 [34-443.5]	382.5 [95.5-497.25]	219 [126-319.5]	NA
Median HIV viral load [IQR], log ₁₀ copies/ml	5.36 [2.55-5.73]	3.23 [1.31-5.57]	4.93 [4.58-5.36]	NA
KSHV infection				
Median KSHV viral load [IQR], log ₁₀ copies/10 ⁶ cells in whole-blood sample	1.94 [1.16-2.85]	4.29 [4.09-4.71]	3.73 [3.70-4.56]	-
Median KSHV viral load [IQR], log ₁₀ copies/10 ⁶ cells in buccal swab sample	-	-	-	3.71 [3.51-4.01]

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455 *HIV: Human immunodeficiency virus; KS: Kaposi's sarcoma; KSHV: Kaposi's sarcoma-associated herpesvirus;*

456 *MCD: multicentric Castleman disease; MSM: men who have sex with men; NA: not applicable; No.: number;*

457 *PEL: Primary effusion lymphoma; PrEP: Pre-exposure prophylaxis*

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462 **Figure 1:** Amino-acid maximum-likelihood phylogenetic tree constructed with PhyML (3.0)
463 of ORF-K1 patients and references sequences for subtype A (A1, A2, A3, A4 and A5), B (B1,
464 B2 and B3), C (C1, C2 and C3) and F (AAX55469_F and AAG01610_F) available from the
465 NCBI database. *The patients' sequences are shown in blue, the reference sequences are*
466 *shown in black and the 3 clusters found are shown in red on the tree. Nodes presenting a*
467 *branch support > 70% (bootstrap analysis with 1000 replicates) are indicated by an asterisk.*

468 *GenBank reference sequence accession numbers: A1: ACS74793, ACS74803 and AAD30529; A2: AAO86800*
469 *and AAD26415; A3: AAB71616; A4: AAD30530; A5: ACS74801, AAG01621 and AAG01597; B1: AAG01622,*
470 *AAG01601 and AAD30531; B2: AAK72680, AAD26369 and AAG01617; B3: AAK72674; C1: AAD26377 and*
471 *AAD30532; C2: ABD52266; C3: AAD30533 and ACY00482; F: AAX55469 and AAG01610.*

472 *GenBank new sequence accession numbers: P149_K1_PEL: MK840448; P076_K1_PEL: MK840449;*
473 *P075_K1_MCD: MK840450; P072_K1_MCD: MK840451; P042_K1_KS: MK840452; P100_K1_PEL:*
474 *840453; P030_K1_KS: MK840454; P062_K1_MCD: MK840455; P044_K1_PEL: MK840456; P073_K1_KS:*
475 *MK840457; P020_K1_KS: MK840458; PrEP_002_K1: MK840459; P095_K1_KS: MK840460;*
476 *P147_K1_MCD: MK840461; P006_K1_KS: MK840462; P077_K1_KS: MK840463; P055_K1_KS: MK840464;*
477 *P007_K1_KS: MK840465; P132_K1_KS: MK840466; P012_K1_KS: MK840467; P078_K1_MCD: MK840468;*
478 *P107_K1_MCD: MK840469; P151_K1_KS: MK840470; P133_K1_MCD: MK840471; P106_K1_KS:*
479 *MK840472; P152_K1_MCD: MK840473; PrEP_001_K1: MK840474; P003_K1_KS: MK840475;*
480 *P009_K1_KS: MK840476.*

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490 **Figure 2:** Comparison, at the time of Kaposi's sarcoma diagnosis, of age (A), KSHV-DNA
491 viral load (B) and immunovirological status (C and D) between patients with KSHV subtype
492 A and patients with KSHV subtype C. **Horizontal lines:** median; **cross:** mean; **boxes:**
493 quartiles 1 and 3; **whiskers:** 95% confidence intervals; **Mann-Whitney U test (p).**

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515 **Figure 3:** Multiple alignment, with Mafft, of partial ORF-K1 amino-acid (aa) sequences
516 (from aa 37 to aa 228) identified as subtype F. *The sequence from Uganda (AAX55469_F;*
517 *shown entirely in color) is compared with sequence described in 2000 from a French MSM*
518 *HIV-infected with PEL (AAG01610_F), and with our five sequences isolated from MSM living*
519 *in France with or without KSHV-associated diseases (P076, P075, P030, P035 and PrEP004)*
520 *and the KSHV sequence of a woman from Congo with multicentric Castleman disease (P072).*
521 *Mismatches are highlighted in various colors, according to the amino-acid concerned, and*
522 *the insertions are indicated by dashes in the other sequences. Hypervariable regions 1 and 2*
523 *are located between aa 54 and 93 and between aa 191 and 228, respectively.*
524 *GenBank new sequence accession numbers: P072_K1_MCD: MK840451; P076_K1_PEL: MK840449;*
525 *P075_K1_MCD: MK840450; P030_K1_KS: MK840454; P035_VR1_KS: MK840478; PrEP_004_VR1:*
526 *MK840477*
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542 **Figure 4:** Nucleotides maximum-likelihood phylogenetic tree constructed with PhyML (3.0)
543 of KSHV-WG patients newly sequenced and reference sequences for subtypes A (A, A3 and
544 A5), B (B1, B3 and B4) and variant C3, available from the NCBI database. *Sanger*
545 *sequencing of ORF-K1 assigned the sequences from P044 and P100 to variants A4 and A1,*
546 *respectively, that from P133 to variant C3, those of P030, P075 and P076 to “Caucasian*
547 *subtype F” and that of P072 to “African subtype F”. The patients’ sequences are shown in*
548 *blue and the reference sequences are shown in black. Nodes presenting a branch support >*
549 *70% (bootstrap analysis with 1000 replicates) are indicated by an asterisk.*
550 *GenBank reference sequence accession numbers: A: AP017458; A3: HQ404500 and KX189629; A5: JQ619843;*
551 *B1: KT271465 and KT271458; B3: KT271460; B4: KT271461 and KT271462; C3: NC_009333_C3_GK18,*
552 *KF588566 and GQ994935.*
553 *GenBank new sequence accession numbers: BC3: MK876731; P030_KS: MK876732; P044_PEL: MK876733;*
554 *P072_MCD: MK876734; P075_MCD: MK876735; P076_PEL: MK876736; P100_PEL: MK876737;*
555 *P133_MCD: MK876738*
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Supplementary Table 1: ORF-K1 reference sequences available on NCBI and used to design DNA-probes for KSHV-enrichment before high throughput sequencing.

Reference number on NCBI	ORFK1 subtype or genotype variant
AF133038	A1
AF130305	A2
U75698	A2
U86667	A3
AF133039	A4
FJ884625	A4
AF178823	A5
AY329023	A5
FJ884624	A5
AF133040	B1
AY042947	B2
AF178818	B2
AY042941	B3
AF178825	B3
DQ309754	B4
AF133041	C1
AF130267	C1
DQ394048	C2
DQ394058	C2
AF133042	C3
GQ994935	C3
AF130268	C3
AF133043	D1
AF133044	D2

AF220292	E
AF220293	E
AY329028	E2
AY953882	F

NCBI: National Center for Biotechnology Information; ORF-K1: open reading frame K1

Supplementary Table 2: Experimental conditions for the amplification of the new KSHV genotype variant by real-time PCR

DNA: Desoxyribonucleic acid; KSHV: Kaposi's sarcoma-associated herpesvirus; Min: minute; PCR: polymerase chain reaction; Sec: second

	Volume (μl) 1 reaction	Final concentration	PCR program			
TaqMan PCR Master Mix 2x	10	1X	Hold	2 min	50°C	1 cycle
Primer Forward (10μM) 5'-TCCTGGTATTGCAACGGAAC-3'	1.8	900nM	Polymerase activation	10 min	95°C	1 cycle
Primer Reverse (10μM) 5'-TCCAAATGCTGTGTGAAGGC-3'	1.8	900nM	Denaturation	15 sec	95°C	40 cycles
Probe (10μM) 5'-GCAGCGATCAGTACCTGTTT-3'	0.4	200nM	Annealing	1 min	60°C	
Extracted DNA	6	-	Hold	∞	4°C	
Total	20	-				

Supplementary Table 3: KSHV-typing results by pathologies and for each patient with KSHV-associated diseases or PrEP users

	KAPOSI SARCOMA				MULTICENTRIC CASTLEMAN DISEASE					PRIMARY EFFUSION LYMPHOMA					PrEP USERS		
No of KSHV-typing (%)	19/41 (46%)				11/12 (92)					4/4 (100)					5/8 (63)		
KSHV-typing	No (%)	Cut and/or oral lesions	Visceral lesions	Native country	Simultaneous or previously KS-diagnosis					Simultaneous or previously KS-diagnosis					No (%)	Native country	Total
					No (%)	No	Cut and/or oral lesions	Visceral lesions	Native country	No (%)	No	Cut and/or oral lesions	Visceral lesions	PEL localization			
A subtype																	
A	-	-	-	-	1/11 (9)	1/1	0	1	F	-	-	-	-	-	-	-	-
A1	1/19 (5)	1	0	F	2/11 (18)	0/2	-	-	2 F	1/4 (25)	0/1	-	-	ascitis	F	-	-
A3	1/19 (5)	1	0	F	-	-	-	-	-	-	-	-	-	-	-	-	-
A4	3/19 (16)	0	3	2 M. Ba 1 F	1/11 (9)	1/1	1	0	M. Ba	1/4 (25)	1/1	0	1	pleural	F	2/5 (40)	1 F 1 S. Am
Total A subtype	5/19 (26)	2	3	-	4/11 (36)	2/4	1	1	-	2/4 (50)	1/2	0	1	-	-	2/5 (40)	-
B subtype																	
B1	0/19 (0)	-	-	-	0/11 (0)	-	-	-	-	1/4 (25)	MD	MD	MD	MD	F	-	-
C subtype																	
C1	1/19 (5)	0	1	M. Ba	1/11 (9)	0/1	-	-	F	-	-	-	-	-	-	-	-
C1-C2	1/19 (5)	0	1	F	-	-	-	-	-	-	-	-	-	-	-	-	-
C3	10/19 (53)	8	2	7 F 1 M. Ba 1 S. Am 1 As	5/11 (46)	4/5	3	1	5F	-	-	-	-	-	-	2/5 (40)	1 F 1 MD
Total C subtype	12/19 (63)	8	4	-	6/11 (55)	4/5	3	1	-	0/4 (0)	-	-	-	-	-	2/5 (40)	-
"F subtype"	2/19 (11)	0	2	1 F 1 S. Am	1/11 (9)	1/1	0	1	F	1/4 (25)	1/1	1	0	pleural	F	1/5 (20)	F

As: Asian; Cut: cutaneous; F: France; KS: Kaposi sarcoma; M. Ba: Mediterranean basin; MD: Missed data; No: Number; PEL: Primary effusion lymphoma; S. Am: South America; Sw: Swiss

Supplementary Table 4

New genotype variant F characteristics

	P030	P035	P075	P076	PrEP004
Age, years old	34	33	50	52	47
Native country	France	Peru	France	France	France
HIV-infection					NA
CD4 count, cells /mm³	85	80	45	162	
HIV-RNA viral load, log₁₀ copies/ml	5.43	1.69	5.69	5.07	
KSHV-infection					
Clinical presentation	Visceral KS: Cutaneous lesions Pulmonary involvement	Visceral KS: Cutaneous lesions Pulmonary involvement Ganglionic involvement	MCD Visceral KS: Digestive involvement Ganglionic involvement	PEL: Pleural involvement KS: Cutaneous lesions	Asymptomatic
KSHV-DNA viral load, log₁₀ copies/10⁶ cells					
- Whole blood sample	5.07	2.83	4.70	4.83	
- Respiratory sample	4.91	4.97			
- Oral swab					5.61
KSHV-typing					
Gene sequenced	ORF-K1	ORF-K1 (VR1)	ORF-K1	ORF-K1	ORF-K1 (VR1)
Subtype	F	F	F	F	F
KSVH whole genome sequencing					
Genome size without repeat region (bp)	137 139	~ 136 000	135 278	137 438	NR
Percentage of coverage	99.36	80.6	98.16	99.01	NR
Depth of coverage (fold)	52	7	108	292	NR

AIDS: acquired immune deficiency syndrome; bp: base pair; HIV: Human immunodeficiency virus; KSHV: Kaposi's sarcoma-associated herpesvirus; NA: not applicable; NR: not realized; ORF-K1: open reading frame K1; VR1: variable region 1





