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This is the author's manuscript

Original Citation:

Availability:

This version is available http://hdl.handle.net/2318/154563 since 2016-02-16T13:02:05Z

Published version:

DOI:10.1159/000358880

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10 May 2024

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***This is an author version of the contribution published on**:*
Questa è la versione dell'autore dell'opera:
Intervirology, Volume 57, Fascicolo 2*,* 2014*,* DOI: 10.1159/000358880.

The definitive version is available at:
La versione definitiva è disponibile alla URL:
http://www.karger.com/Article/FullText/358880

1**Detection of herpesviruses 1-6 and community-acquired respiratory viruses in patients with**

2**chronic rhinosinusitis with nasal polyposis.**

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10**Running title:** herpesviruses and CARV in nasal polyposis.

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18**Key words:** cytomegalovirus; Epstein-Barr virus; human herpesvirus 6; community-acquired

19respiratory viruses; nasal polyposis; functional endoscopic sinus surgery.

20**ABSTRACT**

21**Objective.** To evaluate the prevalence of human herpesviruses 1-6 and community-acquired 22respiratory viruses (CARVs) in specimens from patients with nasal polyposis undergoing functional 23endoscopic sinus surgery (FESS) and investigate the potential clinical role.

24**Methods.** Viral occurrence was evaluated by molecular methods in polyps, turbinate mucosa, pre- 25and post-operative scraping specimens from 35 consecutive patients at different time points in 26relation to FESS.

27**Results.** Overall, 21 patients (60%) were positive to at least one virus in at least one specimen; in 28particular, 12.1% of all specimens for HHV-6 (3/35 polyps, 11/31 turbinates, 1 pre-surgical 29scraping) and 10.5% for EBV (8/35 polyps, 3/31 turbinates, 1/29 pre- and 1/29 post-surgical 30scraping), followed by CMV and HSV-1 (both 1.6%; 1/35 polyps, 1/29 post-surgical scraping and 312/35 polyps, respectively). EBV-positivity tended to be higher in polyps, as well as HHV-6 in 32adiacent healthy turbinate mucosa, although no significant association was found. Only one pre- 33operative cytological specimen was positive to parainfluenza virus-1.

34**Conclusion.** No association between the development of nasal polyps, herpesviruses and CARVs 35seem to exist. However, the higher EBV frequency in polyps could suggest a causative role or 36persistence in the inflammatory lymphoid tissue.

37**INTRODUCTION**

38Nasal polyps are a common chronic disease of nasal and paranasal sinus mucosa, which affects 39approximately 4% of general population. These benign lesions are characterized by inflammation-40induced mucosal swelling, inflammatory cell infiltration, and subepithelial edema. Nasal polyps are 41usually associated with chronic rhinosinusitis (CRSwNP) and most common symptoms are 42obstruction, rhinorrhea, anosmia, facial pain and headache [1]. Medical therapy consists of intra-43nasal steroids [2] and antibiotics [3]; nevertheless functional endoscopic sinus surgery (FESS) is 44often necessary, despite of 70% chance of recurrence [4]. Pathogenesis and molecular mechanisms 45underlying CRSwNP are poorly known; several factors have been investigated, including Kirsten 46rat sarcoma (K-*RAS*) codon 12 mutations/increased expression [5], elevated expression of Vascular 47endothelial growth factor (*VEGFA*) and Transforming growth factor-B1 (*TGF*B1) [6,7], as well as 48clinical features, including allergy, asthma, immunodeficiency and chronic sinus infections [8,9]. 49Viral infections have been hypothesized to play a role in the pathogenesis, progression and 50recurrence of CRSwNP [10]. While human papillomavirus has been associated rather to neoplastic 51lesions [11-13], very few studies have investigated the role of herpesviruses and community-52acquired respiratory viruses (CARVs) with no definitive conclusions [14]. The *Herpesviridae* 53family encompasses several DNA viruses, that are able to establish lifelong latent infections and 54reactivate in immunocompromised conditions; in particular, human herpesviruses 1-6 (including 55HSV-1 and 2, VZV, EBV, CMV) are highly seroprevalent and have been associated to upper 56airway infections.

57The aim of this study was to evaluate human herpesviruses 1-6 and CARVs prevalence by 58molecular methods in nasal polyps, adjacent inferior/middle turbinates, pre- and post-operative 59nasal scraping from patients undergoing FESS.

MATERIALS AND METHODS

Study population consisted of 35 consecutive patients (M/F, 25/10; mean age±standard deviation, 50.3±15.4 years; range, 23-77) with CRSwNP undergoing FESS between September 2011 and April 2012 (Table 1). All the patients gave their informed consent and the study was approved by the institutional review board. Diagnosis of CRSwNP was made on the basis of European Position Paper on Rhinosinusitis and Nasal Polyps (EPOS) 2012 criteria [1]. In details, diagnostic criteria included inflammation of the nose and the paranasal sinuses characterized by two or more symptoms, one of which being either nasal blockage/obstruction/congestion or nasal discharge (anterior/posterior nasal drip); facial pain/pressure; reduction/loss of smell for ≥12 weeks; supported by endoscopic signs of nasal polyps, mucopurulent discharge, oedema/mucosal obstruction and/or computed tomography changes. All patients were affected by CRS with multiple polyps arising from the middle turbinate, middle meatus, or ethmoidal sinuses and were classified as grade II-III according to the Mackay and Lund system [15]. Pediatric subjects, HIV-seropositive individuals, patients with cystic fibrosis, immotile cilia syndrome, allergic fungal rhinosinusitis and inverted papilloma were excluded. The following time points were considered: T_0 (one month pre-surgery) clinical history for allergy and asthma (previous investigation by prick test) and pre-operative cytological specimen; T_1 (surgery) collection of two bioptic samples of polyps and the adjacent inferior/middle turbinates without polyposis; T_2 (one month post-surgery) post-operative cytological specimen (Figure 1). Polyp specimens were collected from maxillary or ethmoid sinus, depending on the involved site; scraping for cytological samples was performed on inferior/middle turbinates. Due to missing sending or inadequacy, only 31, 29, and 29 turbinates, pre- and post-operative scraping specimens were available, respectively, for an overall number of 124 samples for molecular testing.

For processing of mucosa tissues, specimens were incubated with 200 µl lysis buffer (Tissue Lysis Buffer, Qiagen, Milan, Italy) by brief vortexing and heating at 100°C for 5 min twice, vortexed

85briefly again, then centrifuged for 1 min at 13,000 rpm at room temperature. Subsequently, a 86mechanical lysis step with the rotor-stator homogenizer Tissue Ruptor was performed. A 200 µl 87aliquot of supernatant, as well as of nasal scraping, was subjected to nucleic acid extraction using 88the Nuclisens EasyMAG platform (Biomerieux, Marcy l'Etoile, France).

89For herpesviruses 1-6 detection and quantification, real-time PCR was performed, using 90commercially available kits (Q-PCR Complete Kit [ELItech group, Milan, Italy]) following the 91manufacturer's instructions, and the 7500 Real-Time PCR System (Applied Biosystems, Monza, 92Italy). Target regions were glycoprotein D and G for HSV-1 and HSV-2, respectively; ORF 29 for 93VZV; EBNA 1 for EBV; exon 4 and UL 123 for CMV; and ORF 13R for HHV6.

94The occurrence of CARVs was investigated using a commercially available multiplex PCR assay 95according to the manufacturer's instructions (RV15 OneStep ACE Detection [Seeplex®; Seegene, 96Seoul, Korea]), targeting sequences of Influenza A and B viruses, RSV type A and B, adenovirus, 97metapneumovirus, coronaviruses 229E/NL63 and OC43, parainfluenza viruses 1-4, rhinoviruses 98A/B/C, enteroviruses, and bocaviruses 1/2/3/4 and the MultiNA System (Shimadzu Corporation 99Italia, Milan, Italy).

100For statistical analysis, the chi square and Fisher's exact tests were applied, as appropriate. A p-101value <0.05 was considered statistically significant.

102

103**RESULTS**

104Results are summarized in Tables 2. Overall, 21 patients (60%) were positive to at least one virus in 105at least one specimen. As regards herpesviruses 1-6, the highest prevalence was found for HHV-6 106(15/124; 12.1%; mean viral load 1620±1837 copies/10^4 cells; median, 820) and EBV (13/124; 10710.5%; mean viral load 88±140; median, 25), followed by CMV and HSV-1 (both 2/124; 1.6%). No 108specimen was positive to HSV-2 and VZV. In Table 2, prevalence and viral load in different sites 109for each virus are reported. Viral load was $\leq 3x10^3$ copies/10^4 cells in all the cases, except for HHV-1106 on a polyp specimen (see below). Considering the type of specimen, EBV was found in eight, 111three, and two polyp, turbinate and cytological (one T_0 and one T_2) specimens, respectively. In one 112patient, EBV was positive in both polyp and turbinate specimens; in another individual in polyp, 113turbinate and post-operative cytological samples. In both cases, the highest viral load was found in 114polyp specimens. HHV-6 was positive in three polyps, 11 turbinates, and one T_0 scraping specimen. 115In two patients, both polyp and turbinate specimens were positive to HHV-6, with the highest viral 116load being detected in the polyp sample (49000 copies/10^4 cells). HSV-1 was found in two polyp 117samples, one also positive to EBV. CMV was found in one polyp (also EBV-positive) and in a T_2 118scraping (other samples from the same patient were negative to herpesviruses 1-6). Although EBV-119positivity tended to be higher in polyps in comparison to other specimens, as well as HHV-6 in 120turbinate mucosa in comparison to other samples, no statistically significant association was found. 121Similarly, no significant association between EBV and HHV-6 positivity on polyps and turbinate 122mucosa, respectively, and clinical features of allergy and asthma was found. As regards CARVs, 123only one pre-operative cytological specimen was positive to parainfluenza virus 1.

124**DISCUSSION**

125In this study, the prevalence of herpesviruses 1-6 and CARVs in polyp, turbinate mucosa and nasal 126cytological specimens from patients undergoing FESS for CRSwNP was investigated.

127Considering herpesviruses, the highest prevalence was found for HHV-6 (12.1%, irrespective of the 128type of specimen), followed by EBV (10.5%). CMV and HSV-1 prevalence was very low, while no 129specimen resulted positive for other herpesviruses. These results are different from those reported in 130previous studies. As regards EBV, old studies reported EBV-DNA qualitative detection by PCR in 13180% of normal nasopharingeal mucosa from Chinese subjects [16,17]. In another study on 13 nasal 132polyps, the same Authors found EBV prevalence of 15%, 69%, and 85% using southern blot 133hybridization, qualitative PCR, and in situ hybridization, respectively [18]; this study evidenced a 134highly different sensitivity with these different methods and lead the author to hypothesized that 135nasal mucosa is a site where EBV persists through a low replicative level in resident lymphocytes. 136More recently, a 35% EBV positivity in 23 nasal polyps was found by qualitative PCR [19]; 137whereas in a study on nasal polyps and hypertrophied turbinates from Hong Kong patients, no 138specimen was positive to EBV in situ hybridization [20]. Taking into account also the different 139methods and particularly the absence of quantitative molecular data in previous studies, it could be 140argued that EBV-positivity in polyps represents its presence in the inflammatory lymphoid tissue. 141This hypothesis could be further supported by the fact that EBV was detected at lower rate in 142healthy tissue (turbinate mucosa) than in polyps, although the difference was not significant, and by 143the fact that viral load was always within an order of magnitude of 10^2 copies/10^4 cells. Although 144EBV can persist in that lymphocytes can be infected by virus released from a lytic EBV infection in 145the nasal mucosa, the fact that EBV is detected in high rate in normal nasopharyngeal mucosa tissue 146(up to 88% in some studies [18]), whereas nasal polyps are much rarer, argues against an EBV 147contribution to polyp development. This is further supported by data on viral load of the present

148study and the low number of EBV+ cells in each positive case described by Tao and colleagues
149[18].

150Only one study investigated HHV-6 prevalence in polyps and inferior turbinates without finding
151any positive specimen [19]. This is in contrast to the present study in which HHV-6 was detected in
152>35% of turbinate mucosa specimens and >8% of nasal polyps. This difference could be due to the
153different methods: quantitative real-time PCR with high specificity and sensibility in the present
154study, traditional PCR with 70 bp amplicon length in the study by Zaravinos. The relatively high
155HHV-6-prevalence in healthy tissues found in our study could be due to its frequent occurrence and
156diffusion in different tissues. HHV-6 seroprevalence is the highest amongst herpesviruses;
157furthermore HHV-6 is the only herpesvirus which is able to integrate its DNA in human genome, as
158it can be detectable in chromosomically integrated status in 0.2-0.8% of general population.
159Persistence of HHV-6 involves both a true latent state and a low-level chronic replication, each
160occurring at different anatomic sites, including nasal mucosa. It is to note that a possible limitation
161of this study is the lack of healthy controls, as well as of normal sinonasal specimens from patients
162with other underlying pathologies.

163Furthermore, we evaluated the presence of CARVs using a multiplex-PCR and found only one
164cytological sample positive to parainfluenza virus-1. These results are in accordance with a previous
165study on 13 sinonasal mucosa specimens from chronic rhinosinusitis patients and two from healthy
166subjects, that resulted negative to a panel of 12 CARVs [21]. Other Authors evaluated the presence
167of picornaviruses in nasal washing and turbinate mucosa from 39 patients affected by chronic
168rhinosinusitis and 27 healthy people and found a 21% rhinovirus positivity in patients, while no
169virus was found in controls [22]. A more recent study investigated CARVs in paranasal sinus
170mucosa and polyps by multiplex-PCR and found a 18% positivity to bocavirus and <2% positivity
171to rhinovirus in 102 tissue samples from 88 patients [23]. In the present study, the lack of CARVs
172detection in any specimen (but the cytological sample) seems to argue against a potential
173involvement of these viruses in this clinical context.

174In conclusion, only EBV and HHV-6 were detected at certain frequency in nasal polyps and 175adjacent turbinate mucosa specimens, respectively, although with no statistical significance. To 176date, the data obtained by the present and other studies seem to argue against a definite role for 177herpesviruses and CARVs in the development of nasal polyps. Future studies should taken in to 178account the relatively higher frequency of EBV detection in polyps, that could suggest a causative 179role in the formation of nasal polyps, as previously suggested by others, or EBV persistence in the 180inflammatory lymphoid tissue which characterizes these lesions.

181

Acknowledgements

Conflict of interests: none.

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1**Table 1. Demographic and clinical features of study population.**

Patients characteristics	**N** **(Total=35)**	**(%)**
Gender (M/F)	20/15	-
Age (mean ± SD; range)	50.3 ± 15.4	-
Allergy	15	42.9
Asthma	8	22.9
Fanily anamnesis		
Asthma/allergy	10	28.6
Nasal polyposis	3	8.6
Previous sinusal surgery	10	28.6
Nasal obstruction (visual analog scale 0-10)		
0-4	5	14.4
5-8	15	42.8
9-10	15	42.8
Anterior discharge	20	57.1
Posterior discharge	13	37.1
Loss of smell (any grade)	32	91.4
Facial pain	11	31.4

2

3**Table 2. Herpesviruses 1-6 detection and viral load on polyp, turbinate mucosa, pre- and post-**
4**operative scraping specimens from patients with chronic rhinosinusitis with nasal polyps**
5**undergoing functional endoscopic sinus surgery.** For each virus the following data are reported:
6raw number and percentage; viral load as mean±standard deviation and median when more than two
7specimens are positive; otherwise, single results. Viral load is expressed as copies/10^4 cells.

8

Samples	**HSV-1**	**HSV-2**	**VZV**	**CMV**	**EBV**	**HHV-6**
(N= 124)						
Polyps (N= 35)	2 (5.7%)	0	0	1 (2.9%)	8 (22.9%)	3 (8.6%)
	25; 19			35	157±197	16580±28076
					70	440
Turbinates (N= 31)	0	0	0	0	3 (9.7%)	11 (35.5%)
					23±3	705±1264
					25	37
Pre-surgical scraping (N=29)	0	0	0	0	1 (3.4%)	1 (3.4%)
					12	4400
Post-surgical scraping (N=29)	0	0	0	1 (3.4%)	1 (3.4%)	0
					20	
	2 (1.6%)	0	0	2 (1.6%)	13 (10.5%)	15 (12.1%)

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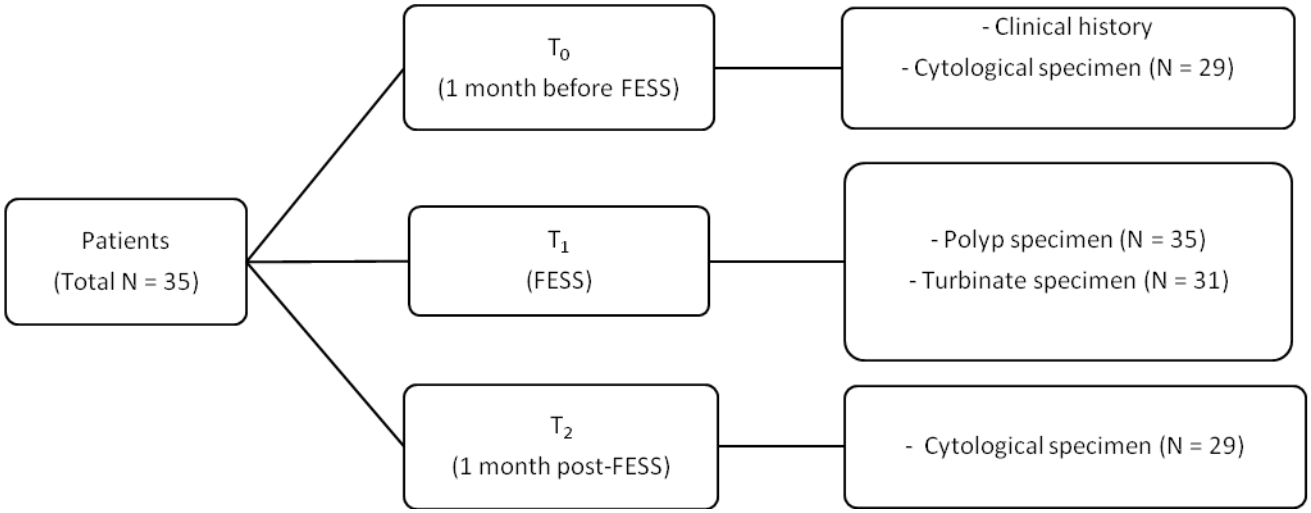

Figure 1. Synopsis of specimen collection in study population. FESS, functional endoscopic sinus surgery.