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ORIGINAL ARTICLE

Enhanced reactivity of peripheral blood immune cells to HSV-1 in primary achalasia

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Abstract

Objective. Achalasia is the best characterized oesophageal motor disorder but the etiology is unknown. The pathology is characterized by a decrease in nitric oxide-producing neurons and the presence of an activated T-cell inflammatory infiltrate in the myenteric plexus that are reactive to HSV-1 viral antigens. These findings are not present in normal controls. The current study compared the reactivity of peripheral blood mononuclear cells (PBMCs) between patients with primary achalasia and normal controls to determine if PBMCs of patients exhibit a similar heightened reactivity to the virus. ***Material and methods.*** Whole blood culture experiments were conducted with heparinized peripheral venous blood obtained from 151 patients with primary achalasia and 118 healthy controls. Whole blood was cultured in the presence of ultraviolet inactivated HSV-1 or conditioned cell culture media. Reactivity of mononuclear cells to viral antigens was quantified by measuring expression of the cytokine gene interferon-γ using Taqman® real-time polymerase chain reaction. Data are expressed as cytokine fold change corresponding to ratio of interferon-γ messenger RNA copies produced in antigen stimulated versus unstimulated cells. ***Results.*** The interferon-γ fold change was higher in cases 61.33 (20.54–217.00) than controls 49.67 (10.05–157.05). Mean fold change difference between cases and controls was 1.66 times (95% confidence interval 1.17–2.34, $p = 0.004$). ***Conclusions.*** These results indicate that the PBMCs of patients with primary achalasia show an enhanced immune response to HSV-1 antigens. The data suggest that there is persistent stimulation of immune cells by herpes simplex virus type 1 (HSV-1) or HSV-1 like antigen moieties.

Key Words: *Achalasia, Herpes simplex virus type 1, immune response, interferon-γ*

Introduction

Although achalasia has been known for more than 300 years, the etiology remains unknown. The pathology is characterized by a loss of nitric oxide-producing neurons in the oesophageal myenteric plexus. What is also observed is the presence of an activated cytotoxic T-cell inflammatory infiltrate [1]. This inflammatory process is believed to cause progressive neuronal damage that leads to the aganglionosis and fibrosis seen in the later stages of achalasia.

Current research supports the potential role of an infectious agent as the initiating event in the pathogenesis of primary achalasia [2–5]. Mononuclear immune cells reactive to Herpes Simplex virus type 1 (HSV-1) antigens have been identified in the oesophageal myenteric plexus of achalasia patients, suggesting that the virus has triggered an immune response at the site [6,7].

HSV-1 possesses certain biological characteristics which makes it a potential candidate in the pathogenesis of primary achalasia. HSV-1 is a double stranded DNA virus and a member of the family *Herpesviridae* which exhibits neurotropism, neurovirulence, the ability to remain in a latent state in neuronal ganglia and the ability to be reactivated. Of the viscera, the oesophagus is the

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most common site of involvement [8]. This may be due to the fact that the virus has a predilection for squamous mucosa and the oesophagus is the only part of the gastrointestinal tract covered with this type of mucosa. Finally, HSV-1 can gain access to the oesophageal myenteric plexus either through the oesophageal mucosa after oral ingestion or through the nervous system after subcutaneous inoculation [9,10].

When infected, the host develops a strong cellular and humoral immunity to the virus [11]. Recovery from acute HSV-1 infections has been shown to depend critically on T-lymphocyte responses and CD8 cytotoxic T-lymphocytes are the predominant effectors that control HSV replication in the nervous system. It is likely that T-lymphocytes control HSV-1 infection by non-cytolytic mechanisms such as secreting cytokines [12]. There is also a strong correlation between interferon-γ levels and the immunological response to HSV-1 infection [13], suggesting that production of this cytokine may be a direct determinant of HSV immune control.

Facco et al. have demonstrated that existence of oligoclonal T-lymphocyte subsets, based on T-lymphocyte receptor characterization, in the myenteric plexus of patients that are reactive to HSV-1 antigens [7]. The current study aims to determine if there is also a systemically heightened immune response to HSV-1 antigens in patients with primary achalasia. The frequency of oral shedding of HSV-1 was also investigated.

Methods

Patient population

A total of 151 patients with primary achalasia were identified from records of oesophageal manometry (1989–2006) performed in the Gastrointestinal Physiology (GIP) Unit, Royal Victoria Hospital, Belfast, Northern Ireland, UK. All cases of primary achalasia in Northern Ireland are diagnosed in the Royal Victoria Hospital GIP Unit as it is the regional centre for oesophageal manometry. A total of 118 controls matched for age and sex were identified and recruited using a population based approach from General Practitioner practices throughout Northern Ireland.

This study was ethically approved (August 2005) by the Office for Research Ethics Committees Northern Ireland. Written informed consent was obtained from all participants and all patients were given an information leaflet detailing the purpose and design of the study.

Determination of lymphocyte count in whole blood

Peripheral venous blood was drawn into a 4 ml Vacuette® EDTA tube and the lymphocyte count of study participants was measured using an automated counter.

Collection of mouth swab for analysis of HSV-1

Saliva specimens for HSV-1 testing were obtained by vigorously swabbing the buccal mucosa with sterile rayon tip swabs (Copan International, Brescia, Italy). The tip of the swab was broken off and immediately placed in lysis buffer (Qiagen Ltd., Crawley, UK) and stored at 2ºC until analysis.

Preparation of HSV-1 viral stocks

HSV-1 virus stocks were prepared in Vero E6 cells and UV inactivated prior to use. Virus titer was determined, as described previously, by the tissue culture infectious dose 50 ($TCID_{50}$) method [14].

Whole blood culture assay

Initially, blood collected was mixed thoroughly by repeated inversion of the lithium heparin tubes. Under aseptic conditions, undiluted whole blood was cultured in 24 well sterile flat bottom untreated polystyrene culture plates (Iwaki, Tokyo, Japan) at a volume of 1 ml per well. For each patient, duplicate wells were stimulated with inactivated HSV-1 (multiplicity of infection: 1 $TCID_{50}$/lymphocyte), phytohemagglutinin (Sigma-Aldrich, Dorset, UK) at a concentration of 2 μg/ml and conditioned cell culture media from uninfected Vero cells. The phytohemagglutinin is a mitogen which stimulated immune cells non-specifically and served as a positive control. Whole blood was incubated in a humidified sealed atmosphere of 5% CO_2.

Nucleic acid extraction

After incubation over 16 hours, 500 μl of whole blood was harvested from each of the six wells and mixed with an equal volume of lysis buffer (Qiagen Ltd., Crawley, UK) in a 2 ml microtube (Sarstedt, Leicester, UK). The final 1000 μl aliquots were snap frozen at –70ºC until use. Total cytokine RNA expressed by immune PBMC was manually extracted from whole blood using the QIAamp® DNA Mini Kit (Qiagen, Crawley, UK). The extracts were subsequently treated with DNase I (New England Biolabs, Hertfordshire, UK) to remove contaminating genomic DNA. Extracted specimens were immediately placed on ice and nested reverse transcription polymerase chain reaction (RT-PCR) performed within

Table I. Primers and probes for detecting interferon-γ mRNA and 18S rRNA.

Primer	Sequence	Product size
First round PCR		
IFN-γ A	5′-TTGGGTTCTCTTGGCTGTTACT-3′	292
IFN-γ B	5′-CTCGTTTCTTTTTGTTGCTATTG-3′	
18S-A	5′-GCTCAGCGTGTGCCTACC-3′	297
18S-B	5′-CCTTGTTACGACTTTTACTTCCTC-3′	
Second round PCR		
IFN-γ C	5′-ATGTAGCGGATAATGGAACTCTT-3′	148
IFN-γ D	5′-ACACTCTTTTGGATGCTCTGG-3′	
18S-C	5′-GTAACCCGTTGAACCCCATT-3′	151
18S-D	5′-CCATCCAATCGGTAGTAGCG-3′	
Probe		
IFN-γ	5′-FAM-TTGAAGAATTGGAAAGAGGAGAGTGACA-TAMRA-3′	
18S	5′-FAM-CATGAACGAGGAATTCCCAGTAAGTGC-TAMRA-3′	

1 h of extraction. HSV-1 DNA was also extracted from mouth swabs using the QIAamp® DNA Mini Kit.

Reverse transcription and first round polymerase chain reaction

A standardized laboratory nested (two round) PCR methodology was utilized to quantify interferon-γ mRNA with normalization to the reference gene 18S ribosomal RNA (18S rRNA). One step reverse transcription of RNA followed by first round PCR of complementary DNA (primers listed in Table I) of whole blood extracts was performed using the Promega Access RT-PCR system (Promega, Southampton, UK) in a 10 μl reaction mixture consisting of: 2 μl extract, PCR buffer, 0.2 mM deoxynucleotide triphosphates (dNTP), 1 mM $MgSO_4$, 1 μM forward and reverse primers, 0.1 U/μl avian myeloblastosis virus reverse transcriptase and 0.1 U/μl *Thermus flavus* DNA polymerase. Reverse transcription was carried out for 10 min at 48°C and the reverse transcriptase was heat inactivated for 3 min at 94°C. PCR cycling conditions were: 3 min at 94°C, followed by 20 cycles of 10 s at 94°C, 10 s at 58°C and 30 s at 72°C on the DNA Tetrad PTC 225 thermal cycler (MJ Research/Bio-Rad, Hertfordshire, UK).

Real-time quantitative PCR

Taqman® quantitative real-time PCR (second round) was conducted on the first round PCR products using the ABI 7000 real-time sequence detection system (Applied Biosystems, Warrington, UK). The primers and probe utilized are detailed in Table I. The PCR reaction mixture (20 μl) composed of the following: 4 μl first round PCR products, PCR buffer, 0.2 mM dNTP, 3.5 mM $MgCl_2$, 0.3 μM 18S primers, 0.2 μM 18S probe or 0.2 μM interferon-γ primers, and probe, 0.1 U/μl *Thermus aquaticus* DNA polymerase and 6-carboxy-X-rhodamine. PCR cycling conditions were: 3 min at 94°C followed by 30 cycles of 30 s at 94°C and 1 min at 60°C. Interferon-γ mRNA and human 18S rRNA levels in samples were analyzed using a method of absolute quantification. Briefly, the raw data generated after each PCR experiment on the ABI 7000 were expressed as cycle threshold (C_T) values.

Absolute quantification using set calibration standards of known concentration was undertaken to quantify the interferon-γ mRNA in each specimen. Serial dilutions of a cloned plasmid DNA complementary to the RNA of interest with a predefined known concentration (copies/μl) were analyzed together with samples in each experiment. The absolute initial quantity of DNA in the samples was derived from a standard calibration curve based on a titration of plasmid copy numbers against C_T. Interferon-γ gene expression was subsequently expressed as cytokine fold change corresponding to the ratio of mRNA produced in HSV-1 stimulated versus unstimulated peripheral blood mononuclear cells (PBMCs). Typical real-time PCR graphs for the quantification of interferon-γ mRNA are shown in Figures 1 and 2.

Analysis of HSV-1 antibodies by ELISA

Herpeselect® 1 ELISA (Focus Technologies Inc., California, USA) was used to detect antibodies to HSV-1 glycoprotein G-1 in the serum of patients and controls.

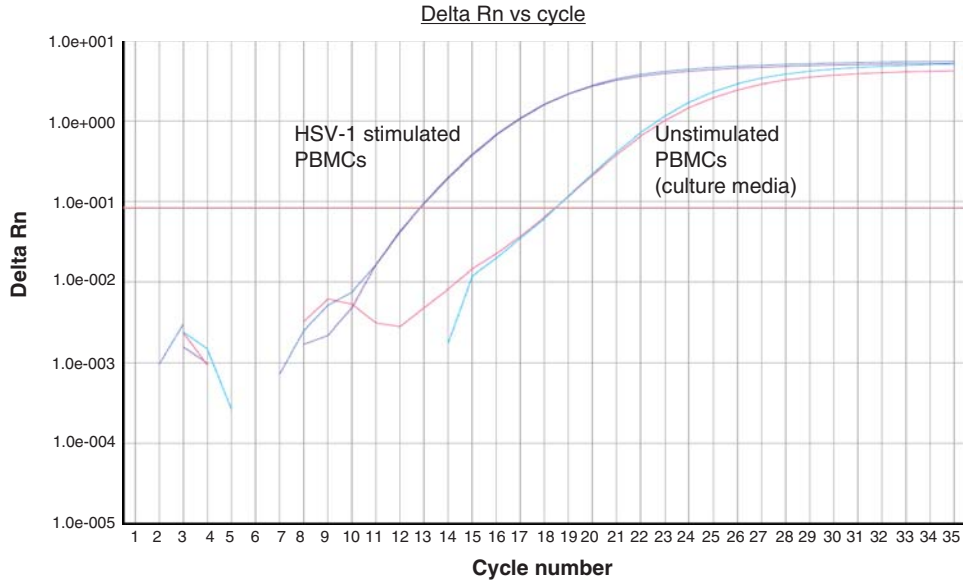

Figure 1. Real-time PCR plot showing interferon-γ mRNA quantification in specimens.

Analysis of mouth swab for HSV-1 DNA using real-time quantitative PCR

Detection of HSV-1 in saliva was performed by standardized laboratory nested (2 round) multiplex PCR as previously described [15]. The primers for HSV-1 are listed in Table II and target the *glycoprotein D* gene. The fluorescent dye SBYR green was utilized to detect amplified HSV-1 DNA on the Roche Lightcycler® real-time machine (Roche Diagnostics Ltd., West Sussex, UK).

Statistical analysis

Data for interferon-γ fold change were positively skewed and expressed as median (interquartile range). Logarithmic transformation was performed prior to all statistical analyses. A multiple linear regression model was used to compare the mean of the log interferon-γ fold change between the cases and controls after adjustment for the following covariates: age, sex, corticosteroid use, HSV-1 antibody status and blood lymphocyte count. Seroprevalence for

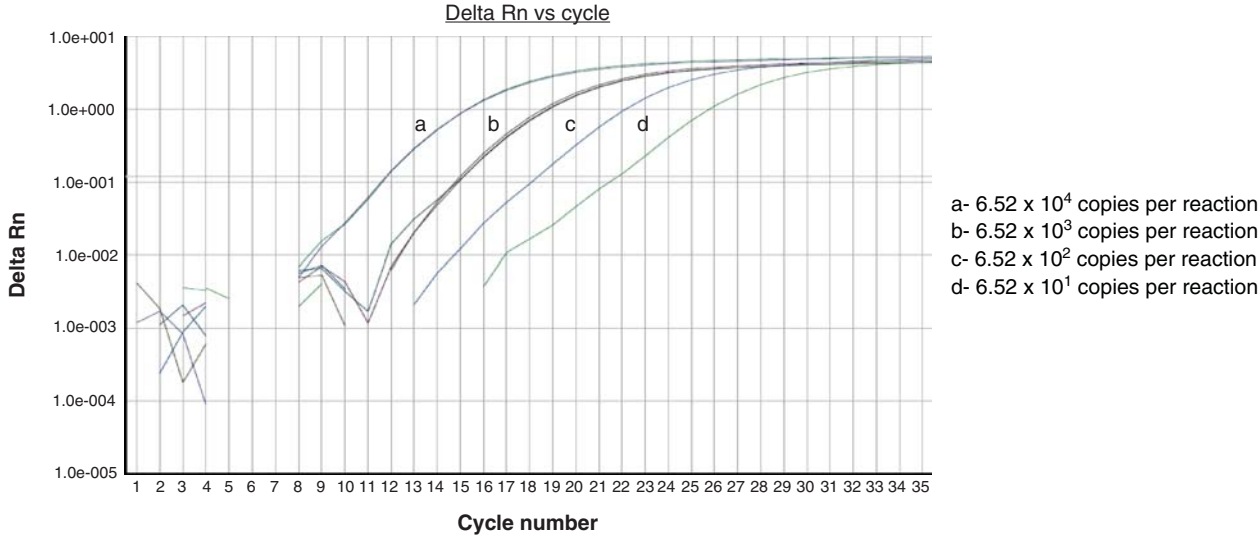

Figure 2. Real-time PCR plot showing log dilution of interferon-γ plasmid standards.

Table II. Primers for detecting HSV-1 DNA.

Primer	Sequence	Product size
First round PCR		
HSV-1 A	5′-ATCACGGTAGCCCGGCCGTGTGACA-3′	221 (Glycoprotein D)
HSV-1 B	5′-CATACCGGAACGCACCACACAA-3′	
Second round PCR		
HSV-1 C	5′-CCATACCGACCACACCGACGA-3′	138 (Glycoprotein D)
HSV-1 D	5′-GGTAGTTGGTCGTTCGCGCTGAA-3′	

HSV-1 was expressed as percentages in each group. The proportion of seropositive patients was compared between cases and controls using the chi-square test. Statistical analysis was carried out using SPSS Version 14 (Chicago, USA).

Results

Clinical characteristics of patients

Patient characteristics are reported in Table III. The mean age of cases at the time of study was 55.4 years (SD 17.1). The mean age of controls was 55.4 years (SD 16.0). The age and sex distribution were similar for both cases and controls.

PBMC interferon-γ gene expression after stimulation with HSV-1 antigens

All participant blood samples contained viable PBMC as confirmed by interferon-γ protein production after non-specific stimulation with phytohemagglutinin (data not shown). Cytokine expression in unstimulated PBMCs was similar in both groups; upon stimulation with HSV-1, the achalasia group demonstrated a higher magnitude of interferon-γ gene expression (Figure 3). The increase in interferon-γ gene expression was 61.33 fold (20.54–217.00) in cases and 49.67 fold (10.05–157.05) in controls (Figure 4). After adjustment for age, sex, corticosteroid use, HSV-1 antibody status and blood lymphocyte count, the geometric mean of cytokine mRNA fold change was 1.66 (95% confidence interval 1.17–2.34) times higher in cases as compared to controls ($p = 0.004$).

HSV-1 seroprevalence

HSV-1 antibodies were detected in most subjects in the study. The seroprevalence of HSV-1 was 82.1% in cases and 83.9% in controls; the difference was not statistically significant.

Oral shedding of HSV-1

The proportion of subjects who shed HSV-1 in saliva was higher in cases (7.9%) than controls (4.3%), but the difference was not statistically significant.

Discussion

In primary achalasia, an inflammatory T-lymphocytic infiltrate reactive to HSV-1 antigens is present in the myenteric plexus [7]. By real-time PCR, we demonstrated the existence of a heightened systemic immune response to HSV-1 antigens in patients with the condition. The changes in interferon-γ gene expression from PBMCs following *ex-vivo* stimulation with HSV-1 antigens were more prominent in patients with primary achalasia than controls. Changes in interferon-γ gene expression levels after

Table III. Clinical characteristics of patients.

	Case	Control
No. of cases	151	118
Mean current age (SD, years)	55.4 (17.1)	55.4 (16.0)
Age range current (years)	16–94	19–88
Mean age at diagnosis (SD, years)	47.1 (17.8)	-
Age range at diagnosis (years)	14–91	-
Median time from diagnosis to participation in study (IQR, years)	6 (1–12)	-
Vigorous achalasia (no., %)*	6 (4)	-
Gender (no., %)		
Male	76 (50.3)	56 (47.5)
Female	75 (49.7)	62 (52.5)

*Vigorous achalasia defined as aperistalsis which is accompanied with increased amplitude contractions and abnormal lower oesophageal sphincter relaxation.

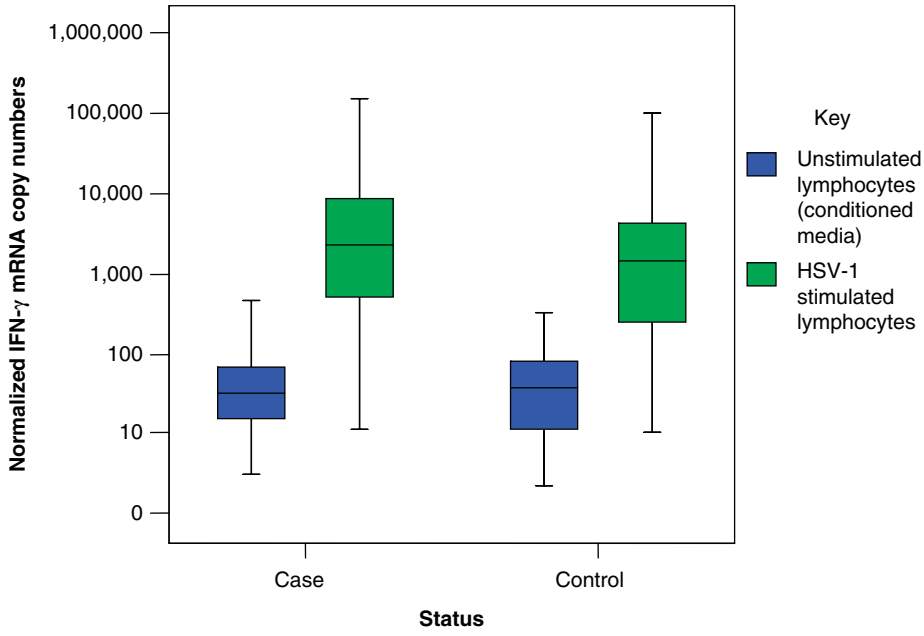

Figure 3. Interferon-γ mRNA (copies per 10^8 18S rRNA copies) in stimulated and unstimulated whole blood plotted on $\log_{10}$ scale.

HSV-1 antigenic stimulation can be regarded as an indicator of T-lymphocyte reactivity to the virus since T-lymphocytes are the main source of the cytokine in adaptive immunity [16,17]. The observed difference in T-lymphocyte activation could be explained by differences in T-lymphocyte immunological memory to HSV-1. The high seroprevalence observed in both groups indicates previous exposure to the virus. After first exposure, a strong antigen-specific cellular and humoral immune response is initiated to achieve viral clearance. Thereafter, most lymphocytes are destroyed by apoptosis. A small fraction of this primed lymphocytic population persists in the circulation (memory cells). Memory cells provide an enhanced immune response to subsequent exposure to the virus. Based on cell surface receptors, memory T-lymphocytes can be categorized into two subsets, known as central (T_C) and effector (T_{EM}) memory cells. T_{EM} cells circulate in the body in an activated state and provide rapid responses, such as production of cytokines, upon secondary contact with a recognized antigen. In contrast, T_C cells are resting cells and require a longer period of activation to exhibit its immune functions [18]. Also, T_{EM} cells express

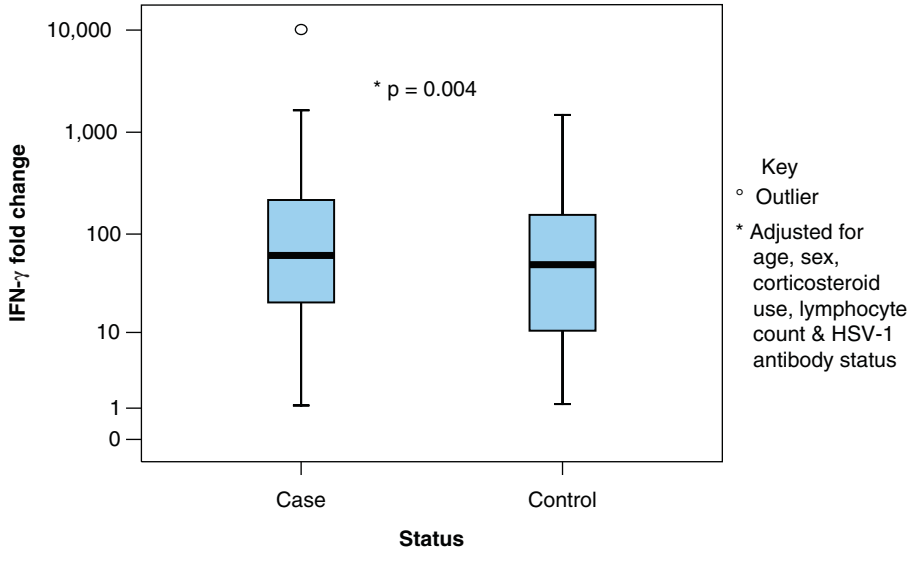

Figure 4. Interferon-γ fold change in cases versus controls plotted on a $\log_{10}$ scale.

surface receptors which allow tissue-specific infiltration whereas T_C cells can only home to secondary lymphoid organs [19].

The maintenance of the relative proportion of cells in each subset of memory T-lymphocytes is regulated by the degree of viral antigenic stimulation. If there is persistent viral antigen stimulation, then a higher proportion will circulate as T_{EM} cells [20]. For example, immunosuppressed transplant patients with a higher Epstein Barr virus (EBV) reactivation burden show a shift of EBV-specific memory cells from T_C to T_{EM} due to persistent viral antigenic stimulation [21]. It is possible that patients with achalasia have a higher proportion of circulating HSV-1 specific T_{EM} memory cells compared to controls leading to the observations seen in the current work. If this is true, then the reasons for this persistent antigenic stimulus must be elucidated.

The ongoing stimulus driving the T_{EM} memory cells could be due to more frequent reactivation of HSV-1 infection in patients with achalasia. Although there is no clinical evidence to suggest that achalasia patients experience more frequent HSV-1 reactivation, it is known that a high proportion of reactivation is asymptomatic. In addition, the current study also observed a higher proportion of achalasia cases that were shedding HSV-1 in saliva than controls, although this did not reach statistical significance. Reactivation of HSV-1 in the facial ganglia or vestibular ganglia has been linked to neuronal damage and the development of Bell's plasy and vestibular neuronitis [22]. If the site of reactivation is in the oesophageal myenteric plexus, the ensuing immune response could lead to progressive neuronal dysfunction or damage. Recent data have also shown that patients with primary achalasia have serum factors, believed to be cytokines, that resulted in a reduction of the population of nitric oxide-producing neurons [23].

An alternative explanation is that the original HSV-1 infection has triggered the development of cell-mediated autoimmunity in patients with achalasia, possibly by molecular mimicry [24]. T-lymphocytes recognize and mount an immune response to self antigens that are identical to HSV-1 antigens. In herpes stromal keratitis, another HSV-1 induced disease, cross-reactive T-cell clones recognized both corneal antigens and a similar HSV-1 virion associated protein [25].

The findings of the current study appear to differ from that conducted by Facco et al. where they found no difference in the proliferation index of PBMCs of patients with primary achalasia compared to control subjects [7]. One possible explanation is that the control population in that study composed of heart-beating cadaveric organ donors who may have been given medications that have altered T-lymphocytic function. Also, their small study population (41 patients and 23 controls) may have inadequate statistical power to detect any differences between achalasia cases and controls. To our knowledge, our study is the largest study to date investigating potential etiological factors in the pathogenesis of primary achalasia utilizing a population based approach.

In conclusion, the current study has provided further supportive data on the potential role of HSV-1 in the pathogenesis of primary achalasia. The heightened immune response seen in PBMCs of patients with primary achalasia could possibly be due to frequent viral reactivation or alternatively, an autoimmune response to HSV-1 like self antigens.

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***Declaration of interest*:** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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