Epstein-Barr Virus Infection in the Edinburgh Student Population

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Declaration

This thesis has been composed by myself. The work included is my own, with contributions from other members of the research group, which have been stated in the acknowledgements and text. The work was conducted in SE Scotland and has not been submitted for any other university degree. With the assent of the Postgraduate Studies Committee the flow cytometry data formed part of a dissertation submitted in partial fulfilment of the second part of the MRCPath examination.

Karen Macsween

In memory of

John Angus Macsween 1939-2006

Abstract

A longitudinal seroepidemiological cohort study of Epstein-Barr virus (EBV) in the Edinburgh student population was conducted between 1999 and 2003. During this time 60 cases of infectious mononucleosis (IM) were recruited at the University Health Centre to join a case control study to investigate the symptoms of IM and its impact on daily activities.

In the longitudinal cohort study a total of 2006 students (1258 female and 748 male) commencing four year degree courses in 1999 or 2000 had their EBV serology determined and completed a lifestyle questionnaire. Overall 1499 (75%) were EBV seropositive at recruitment. Female students were more likely than male students to be seropositive (79% vs. 69%, $p<0.001$), as were, students with siblings (75%, compared to those without 66%, $p=0.023$), and those reporting prior residence in a tropical country (81%, $p=0.003$). Students reporting a prior sexual relationship were more likely to be seropositive (83%, $p<0.001$). Repeat serological testing of 241 initially seronegative students undertaken at the start of their fourth year of study showed that 110 (54%) had seroconverted over the intervening three year period. The proportion experiencing IM was 25%.

Of the 60 IM cases recruited to the case control study, the great majority 56/60 (93%) had either sore throat or complaints relating to lymphadenopathy when presenting to their GP. Few students complained of fever (13%), and some had presentations that included atypical symptoms including gastrointestinal complaints 8% (predominantly vomiting), headache 10%, fainting 10% or cough 7%. Fatigue was experienced by all cases during the period of their most severe symptoms, although the severity and duration varied considerably. Female students experienced more severe and prolonged fatigue (median 125 days) than males (median 54 days, $p=0.002$). Overall 73% of students missed university classes because of IM. Female students were more likely to miss timetabled university classes (median 17 hours) than males (median 2 hours, $p=0.004$) and were more likely to discontinue their studies completely (16% vs. 0%, $p=0.055$). All cases, but only 66% of the control group students reported any sexual contact, including kissing, in the preceding three months ($p<0.001$). Analysis of lymphocyte subsets in IM cases and controls demonstrated prolonged perturbations of some subsets following IM, with elevated natural killer and gamma delta T-cell populations 5 or more months after diagnosis.

Strain typing was performed on EBV DNA obtained from IM cases and their partners, friends and flatmates. Matching strains were found in 9/11 (82%) IM and partner pairs, but in only 3/17 (18%) of non-sexual contacts of IM cases ($p=0.001$).

The study demonstrates that students with IM are likely to be sexually active, and may therefore have additional health care needs. IM in university students is associated with prolonged clinical, immunological, and social consequences. Female students are more likely than males to experience a protracted fatigue state and to miss university classes.

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Contents

Tables

Table 26 Comparison of lymphocyte subset ranges in controls with other published data. 147

Figures

Abbreviations

AIDS	Acquired immunodeficiency syndrome
ALKP	Alkaline phosphatase
ALT	Alanine aminotransferase
*Bam*H1	Restriction endonuclease of *Bacillus amyloliquefaciens*
BARTs	*Bam*H1 A rightward transcripts
bp	basepair
BCR	B cell receptor
BL	Burkitt's Lymphoma
BLPD	B-cell lymphoproliferative disease
BSA	Bovine serum albumin
CAEBV	Chronic active EBV
CD	Cluster of differentiation
CI	Confidence interval
CMV	Cytomegalovirus
CNS	Central nervous system
CSTs	Complementary strand transcripts
DNA	Deoxyribonucleic acid
DZ	Dizygotic (twins)
EA	Early antigen
EBER	EBV encoded RNA
EBNA	Epstein-Barr nuclear antigen
EBV	Epstein-Barr virus
FBC	Full blood count
FCM	Flow cytometry
FCS	Foetal calf serum
γδ	Gamma-delta (T-lymphocyte)
g	Gram
GGT	Gamma Glutamyl transferase
gp	Glycoprotein
GP	General practitioner
HAART	Highly active antiretroviral therapy
Hb	Haemoglobin

HBSS	Hanks Balanced Salt Solution
HD	Hodgkin's Disease
HIV	Human immunodeficiency virus
HHV	Human Herpes Virus
HLA	Human leucocyte antigen
HRS	Hodgkin Reed Sternberg
HSV	Herpes simplex virus
HV	Herpes virus
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IM	Infectious mononucleosis
IR	Internal repeat
Kb	Kilobase
KSHV	Kaposi's Sarcoma associated herpes virus
l	Litre
LANA	Latency associated nuclear antigen
LCMV	Lymphocytic choriomeningitis virus
LCS	Longitudinal cohort study
LFT	Liver function tests
LMP	Latent membrane protein
LP	Leader protein
m	Meter
M	Molar
µg	Microgram
MHC	Major histocompatibility complex
µl	Microlitre
mm	Millimetre
mol	Mole
mRNA	Messenger ribonucleic acid
MS	Multiple sclerosis
MZ	Monozygotic (twins)
n	Nano

NK cell	Natural killer cell
NHL	Non-Hodgkin lymphoma
NPC	Nasopharyngeal carcinoma
OD	Optical density
p	Pico
PBMC	Peripheral blood mononuclear cell
PCR	Polymerase chain reaction
PTLD	Post-transplant lymphoproliferative disease
RBC	Red blood cell
RNA	Ribonucleic acid
s	Second
SAP	SLAM associated protein
SH2DIA	Src homology 2 domain containing protein
SLAM	Signalling lymphocytic activation molecule
TCR	T cell receptor
TNF	Tumour necrosis factor
TP	Terminal protein
U	Unit
UHC	University Health Centre
UL	Unique long
US	Unique short
UV	Ultra violet
v/v	Volume per volume
VCA	Viral capsid antigen
VL	Viral load
VZV	Varicella zoster virus
w/v	Weight per volume
XLP	X-linked lymphoproliferative disease

Symbols

α	Alpha
β	Beta
δ	Delta
γ	Gamma
μ	Mu
χ	Chi
$>$	Greater than
$\geq$	Greater than or equal to
$<$	Less than
$\leq$	Less than or equal to

Aim

The present study was undertaken to investigate the current epidemiology of EBV infection in a student population, the risk factors for symptomatic seroconversion and the clinical and social impact of symptomatic infection.

1 Herpes Viruses

1.1 Herpes Virus Family

Herpes viruses are widespread animal viruses found in diverse species ranging from molluscs to man. Membership of the family is based on the distinct virus morphology and shared biological features, which include, the ability to remain latent in their hosts, destruction of the infected cell when virus progeny are produced, synthesis of viral DNA and assembly of the viral capsid in the host cell nucleus, and the production of a large number of viral enzymes (Minson *et al*, 2000; Pellet & Roizman, 2007). There is marked sequence diversity between the viruses of homoiothermic (warm blooded birds and mammals) and poikilothermic (cold blooded) animals, although the herpesvirus structure is conserved. The herpes viruses have large double stranded DNA genomes encased in an icosohedral nucleocapsid, composed of 162 capsomers, which varies little in size between host species, despite the variable size of the enclosed genome. The capsid is coated with a layer of proteins known as the tegument and this is surrounded by a lipid bilayer envelope, derived from the host cell which contains virus-encoded glycoproteins. Interactions between cellular receptors and viral glycoproteins trigger envelope-membrane or cell-cell fusion. Three glycoproteins, designated gB, gH and gL, are thought to be essential for fusion in all herpes viruses, although additional viral glycoproteins may be required as fusion ligands (e.g. gD) and as binding ligands (e.g. gC) (Spear & Longnecker, 2003).

1.2 Classification of Herpes Viruses

The family *Herpesviridae* is divided into three subfamilies- alpha, beta and gamma which all share features of herpes virus (HV) biology, including a high incidence of asymptomatic infections, thought to reflect the long co-evolution with their respective hosts, and, the establishment of latency with episodes of reactivation, which allows horizontal transmission to new hosts. The host-virus relationship is finely balanced and disease states may occur, particularly in situations of impaired host immunity or if infection of an unusual host occurs. Initially the classification was based upon biological properties e.g., permissive cell types, length of the reproductive cycle in cell culture and oncogenic potential, however, classification is now based on the organisation and content of the genome. Generally alpha and beta herpes viruses are not oncogenic, although notable exceptions exist, particularly

Marek's Disease HV of chickens. Gamma herpes viruses establish latent infection in lymphocytes and are associated with lymphoproliferative disease.

1.3 Evolution of Gamma Herpes Viruses in Primates

Prior to the year 2000 EBV-like B-lymphotropic gamma herpes viruses had been found only in Old World primates. The discovery of EBV-like sequences in the peripheral blood of squirrel monkeys, *Saimiri sciureus,* (Wang *et al*, 2001) and in spontaneous B-cell lymphomas of New World common marmosets, *Callithrix jacchus,* (Cho *et al*, 2001; Ramer *et al*, 2000), implied that primates and EBV-like gamma-herpes viruses had been evolving together prior to the split of New and Old world primates which occurred 35 – 47 million years before the present day (Kumar & Hedges, 1998; McGeoch, 2001).

Comparison of conserved herpes virus genes permits classification into subfamilies and the study of phylogenetic relationships. Gamma herpes viruses are subdivided into two genera the lymphocryptoviruses (γ-1), found only in primates, and rhadinoviruses (γ-2) which are found in a broader range of mammalian species. Both genera share aspects of genome organisation and some γ–herpes virus specific genes, including the transcriptional activator known as BRLF1 in EBV; an anti-apoptotic Bcl-2 homologue; integral membrane proteins that in EBV are designated latent membrane proteins 1 and 2 (LMP1 and LMP2); and, genes that are analogous in function e.g., Epstein-Barr virus nuclear antigen 1 (EBNA1) and latency-associated nuclear antigen (LANA) which allow the persistence of the genome as an episome in latently infected cells (Kieff & Rickinson, 2007; McGeoch, 2001).

Lymphocryptoviruses, unlike rhadinoviruses, immortalise B-lymphocytes and to this end γ-1 viruses share genes associated with cell immortalisation. Both γ-1 and γ-2 have homologues of cellular genes e.g. the interleukin (IL)-10 homologue in EBV and the IL-6 homologue in Human Herpes Virus 8 (HHV8).

1.4 Human Herpes Viruses

The human herpes viruses (HHVs) are a group, currently numbering eight, endemic in human populations (Table 1). EBV was the fourth member of the group to be discovered, which also includes herpes simplex viruses 1 and 2, varicella zoster virus, cytomegalovirus, HHV6A, HHV6B and HHV7, aetiological agents of erythema subitum, and HHV8 or Kaposi's Sarcoma associated herpes virus (KSHV). Human herpes viruses are represented in all three subfamilies (alpha, beta and gamma) of the *Herpesviridae.* Following primary infection they all establish persistent infection resulting in periodic reactivation and shedding of infectious virus. The extent of reactivation is influenced by the host immune system.

Table 1 Summary of Human Herpes Viruses

Systematic name	Virus common name	Abbrev. form	Illness associated with primary infection	Illnesses associated with reactivation or persistence, including tumours	Genus	Subfamily	Genome length kbp	G+C content	Location of latent infection
HHV1	herpes simplex type 1	HSV-1	Oral and ocular herpes Gingivostomatitis, herpetic whitlow, encephalitis	Oral, ocular and genital herpes Encephalitis	*Simplexvirus*	α	152	68%	Neuronal cells
HHV2	herpes simplex type 2	HSV-2	Genital herpes	Genital herpes	*Simplexvirus*	α	155	70%	Neuronal cells
HHV3	varicella zoster virus	VZV	Chickenpox	Shingles	*Varicellovirus*	α	125	46%	Posterior root ganglion cells
HHV4	Epstein-Barr virus	EBV	Infectious mononucleosis	Oral hairy leukoplakia BL, NPC, HD, PTLD	*Lymphocryptovirus*	γ–1	172	60%	B-lymphocytes

HHV5	cytomegalovirus	CMV	CMV mononucleosis Congenital infection, pneumonitis, retinitis, colitis in immunosuppressed	Congenital infection, pneumonitis, retinitis, colitis in immunosuppressed	*Cytomegalovirus*	β	230	57%	Mononuclear cells
HHV6A		HHV6A	Exanthem subitum	Encephalitis; colitis pneumonitis in immunosuppressed	*Roseolovirus*	β	159/170	43%	Mononuclear cells
HHV6B		HHV6B	Exanthem subitum Infectious mononucleosis-like illness	Encephalitis, colitis pneumonitis in immunosuppressed	*Roseolovirus*	β	162/168	43%	Mononuclear cells
HHV7		HHV7	Exanthem subitum	Hepatitis, fever in immunocompromised	*Roseolovirus*	β	145	36%	$CD4^+$ T-lymphocytes
HHV8	Kaposi's Sarcoma herpes virus	KSHV		Kaposi's Sarcoma	*Rhadinovirus*	γ–2	170/210	59%	B-lymphocytes

BL Burkitt's lymphoma; NPC nasopharyngeal carcinoma; HD Hodgkin's Disease; PTLD post transplant lymphoproliferative disease

Adapted from (Britt, 2005; Ganem, 2007; Mocarski Jr *et al*, 2007; Pellet & Roizman, 2007; Roizman *et al*, 2007; Yamanishi *et al*, 2007)

The prevalence of the individual viruses varies from universal infection at an early age, as is seen with HHV6B, to being an infection which mainly affects restricted geographical, ethnic or behavioural groups with HHV8. Infection with HHVs occurs via mucosal surfaces, although other routes of transmission are also seen. Cytomegalovirus (CMV), varicella zoster virus (VZV) and herpes simplex viruses (HSV) 1 and 2 are associated with vertical infection of the foetus; and EBV, CMV and HHV8 have been transmitted by blood transfusion and / or organ donation.

1.5 The Discovery of EBV

Denis Burkitt, a surgeon working in Uganda after the Second World War, became interested in an unusual tumour of the maxilla or mandible in young children in equatorial Africa. The tumour was often multifocal at presentation, with deposits in the liver, adrenals or kidneys, and responded poorly to surgery although appeared to regress with chemotherapy, which was in short supply. Following a number of "medical safaris" he recorded that the tumour was geographically restricted and related this to altitude, temperature and rainfall (Burkitt, 1958; Burkitt, 1962a; Burkitt, 1962b; Burkitt, 1983; Olweny & Nkrumah, 1985). In 1961 he presented his early findings at a lecture in The Middlesex Hospital London where Anthony Epstein, a virologist, was in the audience. Surmising that the tumour might be caused by an insect-vectored oncogenic virus he asked Burkitt to send tumour samples to his laboratory. For the next two years Epstein, together with Bert Achong and a PhD student, Yvonne Barr, tried to grow the samples in tissue culture at The Middlesex Hospital without success. Epstein relates the delayed arrival of one tumour specimen which appeared turbid, and thinking this would represent bacterial contamination, he decided to examine the specimen microscopically before discarding it. Instead of bacterial contamination he found large numbers of viable-looking tumour cells which reminded him of the successful culture of murine lymphoid cells he had seen previously at Yale University. Thus the sample instead of being discarded became the first Burkitt's Lymphoma (BL)-derived continuous cell line. After almost three months of culture there was sufficient material to allow a portion to be prepared for electron microscopy which revealed virus particles with the characteristic morphology of a herpesvirus (Epstein, 1999; Epstein *et al*, 1964; Epstein, 1985). Collaboration with Gertrude and Werner Henle in Pennsylvania confirmed that the newly discovered virus was distinct from the then known human herpes viruses HSV, VZV and CMV (Henle & Henle, 1966). Later when EBV was shown to be an almost universal infection of humankind, additional co-factors were sought to explain the association of EBV with BL. This led to the observation of the correlation between malaria intensity and tumour

incidence (Burkitt, 1983).

In the US, shortly after the description of EBV, the Henles were screening sera from patients with unidentified viral infections when one of their research technicians contracted infectious mononucleosis (IM), a disease then of unknown aetiology, characterised by sore throat, lymphadenopathy and fever (see 2.1.1). Investigation showed evidence of seroconversion to Epstein's newly discovered virus (Henle *et al*, 1968).

1.6 Epidemiology of EBV

1.6.1 Socio-economic and Geographical Factors

After the finding of EBV in cell lines derived from BL it became apparent that, unlike BL, EBV infection was in fact widespread, although the timing of primary infection showed marked geographical and socio-economic variation. Seroepidemiological studies in Africa demonstrated very early EBV acquisition with 80-100% of infants infected by their first birthday (Biggar *et al*, 1981; Kafuko *et al*, 1972). Urban African children residing in more affluent homes showed slightly more delayed EBV acquisition, in some cases until the third birthday (Biggar *et al*, 1981). Studies in Europe and the USA have shown later acquisition of EBV with only 39% of children aged between 5 and 14 having serological evidence of infection in a laboratory based UK study (Pereira *et al*, 1969) and 50% acquisition by 2 years of age in a hospital based study in Philadelphia (Fleisher *et al*, 1979). A Danish study found an association between positive EBV serology and the occupation of the head of the household. Where the head of the household followed an "unskilled" occupation or was unemployed 54% of children had antibodies as opposed to 34% of children from a "skilled" or "professional" background ($p<0.05$) (Hesse *et al*, 1983).

Persistence of EBV seronegativity into the teenage years has been associated with higher socio-economic status as assessed by the occupation of the head of the household (Dan & Chang, 1990), the income of the household (Hallee *et al*, 1974) or residing in owner-occupied housing (Dan & Chang, 1990). The association of earlier EBV seroconversion with the presence of siblings has not been a constant finding; Dan and Chang (1990) and Hallee *et al* (1974) found no association, whilst an Australian study found an excess of seroconversions amongst siblings (Lai *et al*, 1975). A study based in Edinburgh primary schools, using a salivary antibody detection method, also failed to find a significant association with numbers of children in the home, but did find significant associations between seropositivity and unemployment or manual occupation of the head of the household; sharing a bedroom; or attending a school in an area with predominantly rented housing estates (Crowcroft *et al*, 1998). The finding of an excess of IM, which reflects

delayed EBV acquisition, in young people from higher socio-economic groups was confirmed in a small study in London which compared the occupation of the head of the household where an IM case arose, with the socio-economic grouping of the surrounding population (Nye, 1973).

1.6.2 Serological Status by Age and Gender

Seroprevalence increases with age, the rapidity of acquisition depending on the affluence of the household and community. In affluent communities where seroconversion is delayed, two peaks of acquisition are seen; one in children under 5 years and a second in the teenage years (Lai *et al*, 1975; Morris *et al*, 2002). A recent UK based study, based on serological samples submitted for diagnostic purposes, showed 35% of children aged 1-4 years old, 54% of 10-14 year olds and 72% of 15-19 year olds had evidence of past EBV infection (Morris *et al*, 2002). In adolescents aged 10-14 years EBV seroprevalence increased at a younger age in females, than males; a finding corroborated by an earlier community-based survey of IM in the US, where the peak incidence of IM in girls occurred two years younger than in males (Heath *et al*, 1972). It has been proposed that the epidemiology of EBV is currently changing in the UK, in parallel with similar changes occurring in the epidemiology of HSV-1, with more EBV seroconversions being delayed into later childhood and adolescence (Morris *et al*, 2002). Similar delays in the acquisition of EBV have recently been described in Japan (Takeuchi *et al*, 2006).

1.6.3 Epidemiology of EBV and Infectious Mononucleosis in Students and Military Populations

The association of EBV seroconversion and IM observed at the Henles' laboratory was confirmed by testing serum samples that had been collected from students at Yale University prior to, and after the onset of IM (Niederman *et al*, 1968). IM was recognized as an important cause of ill-health in populations with large numbers of young people, including universities and the military. In the 1960s IM was noted to be the third most common cause of days lost to hospitalization in the US Air Force and fourth most common in the Navy (Evans, 1970). In the 1960s and '70s the epidemiology in military recruits and students was further investigated. Low seroconversion rates were found in US marine recruits and students at The University of Hong Kong (2-7% per year, (Dan & Chang, 1990; Lehane, 1970)) whilst high rates were observed at Yale University (43-65% per year (Niederman *et al*, 1970; Sawyer *et al*, 1971)). Rates of IM varied from zero in Peace Corps Volunteers and Hong Kong Students (Dan & Chang, 1990; Niederman *et al*, 1970) to 13% of EBV seronegative students per year at Yale University (Sawyer *et al*, 1971). The proportion of EBV seroconversions that resulted in documented illness also varied widely; from none (Dan

& Chang, 1990; Niederman *et al*, 1970) to 74% (Sawyer *et al*, 1971). These prospective studies are summarised in Table 2.

A study of 19 universities in the US showed a particularly low incidence of IM at the University of Hawaii which was not thought to be adequately explained by the low (13%) prevalence of EBV-seronegative students. Possible additional explanatory factors were the high proportion of Asian students, the high proportion of students living with parents or relatives, which resulted in low use of the university student health facilities, and which may also have resulted in other social, sexual or health-care seeking behavioural differences in the students, although these were not explored (Chang *et al*, 1979). A study amongst Chinese students in Hong Kong showed that a large majority (93%, 71/1039) of students were seropositive at university entry, and that all 16 subsequent EBV seroconversions in the study were asymptomatic (Dan & Chang, 1990). Factors associated with seronegativity at university entrance in this study were non-manual occupation of the head of the household, and, residing in privately owned, as opposed to public housing. Seroconversion occurred in 26% (16/62) of the seronegative of students in one year, of whom 14/16 (88%) had not engaged in "deep kissing that resulted in the exchange of saliva" (See 1.9.4 and 1.9.5). In fact only three seronegative students admitted to kissing, and of these 2 seroconverted. Later sociological studies have highlighted differences in the sexual behaviour of Asian and Caucasian students (Huang & Uba, 1992; Meston *et al*, 1996).

At West Point Military Academy race was found to be a significant determinant of seropositivity at entrance with 81% of black cadets and 62% of white cadets being seropositive (Hallee *et al*, 1974). Income of the family was also significant, with cadets from the lowest income families more likely to be seropositive than those from the highest. The study by Sawyer *et al* at Yale differs from other IM studies in that all EBV seroconversions were associated with some symptoms, and of these 17/23 (74%) fulfilled a very strict case definition for IM (Sawyer *et al*, 1971).

Hallee *et al* (1974) and Sawyer *et al* (1971) were unable to demonstrate an increased incidence of IM or EBV infection amongst susceptible roommates of IM cases.

Table 2 Summary of prospective studies of infectious mononucleosis

	Location	Gender	EBV seronegative at entry	Seroconversion rate per year	IM rate in seronegatives per year	Proportion of seroconversions symptomatic	Comments
(Hirshaut *et al*, 1971)	Cornell University	Not stated	25% (200/800)	Not stated	3.8% (15/200 in 2 years)	Not stated	Abstract only
Lehane 1970	American Marine Recruits	Male	18.1% (117/648)	6.9% in training (calc) 2.2% in Vietnam (calc)	Not stated hospitalisations for IM in Vietnam 0.2% per year	9% (based on rate of hospitalizations for IM in Vietnam troops)	No individual follow-up of IM cases
Niederman *et al* 1970	Yale University	Male	64.7% (97/150)	11.1%	4.4% per year	65.1% 28/43	
Niederman *et al* 1970	Peace Corps Volunteers	Not stated	23.8% (39/164)	26.7% 8/30	No IM cases	No IM cases	
Sawyer *et al* 1971	Yale University	Male	49% (175/355)	17.5% per year (23/175, 13.1% in nine months)	13% per year calc 9.7 per 100 in 9 months	73.9% definite IM 100% had some symptoms	Blood taken at every illness
University Health Physicians 1971	6 UK Universities	Male & Female	42.7% (622/1457)	20.7% per year (60/496, 12% in 7 months)	6% per year (22/622, 3.5% in 7 months)	37% 22/60	Overseas students 81/96 84% pos Follow-up incomplete
Hallee *et al* 1974	US Military Academy	Not stated presumed male	36.5% (511/1401)	20.7% overall 45.9% in 4 years 12.4% in first year 54/437	3.4% (15/437) Definite + retrospective 6.2 %	27.8% (15/54 definite IM cases) suspected in retrospect for additional 12 i.e. 50%	
Dan and Chang 1990	Chinese University Hong Kong	51% male	6.8% (71/1039)	25.8% 16/62	No IM cases	No IM cases	Kissing 2/16 seroconverters

1.7 Epidemiology of HSV and CMV

The other human herpes viruses share aspects of epidemiology with EBV. The epidemiology of herpes simplex viruses and cytomegalovirus, in particular, has been extensively studied. These viruses show horizontal transmission amongst young children, which is mostly asymptomatic, and evidence of sexual transmission in adults.

1.7.1 Herpes Simplex Viruses

HSV-1 and HSV-2 are usually transmitted by different routes and typically affect different areas of body. Like EBV, HSV-1 is usually acquired horizontally in childhood, through contact with oral secretions, and early seroconversion is associated with poor socio-economic status (Whitley & Roizman, 2001). Oral or genital lesions may arise with HSV-1 depending on the mode of transmission. In the UK, the seroprevalence of HSV-1 has been falling in children and in student populations (Best *et al*, 1994; Vyse *et al*, 2000), and the proportion of symptomatic sexually acquired HSV-1 has been rising (Scoular *et al*, 2002). It has been proposed that similar epidemiological trends will apply to EBV in view of the oral salivary transmission route that they both share in childhood (Morris *et al*, 2002).

HSV-2 is usually sexually transmitted and typically results in genital lesions (Nahmias *et al*, 1990; Whitley & Roizman, 2001). In a cross-sectional survey of US undergraduates increasing seroprevalence of HSV-1 and HSV-2 were each associated with a longer duration of sexual activity in years, and with a prior history of sexually transmitted infection (Gibson *et al*, 1990). A large cross-sectional survey in the US investigating HSV-2 seroepidemiology found significant positive associations with female gender, non-Hispanic black ethnicity, increasing numbers of lifetime sexual partners (Xu *et al*, 2006). Interestingly this study reported a fall in HSV-2 seroprevalence in the period 1999-2004 compared with the period 1988-1994 which was concentrated in the younger age groups. The proportion of participants reporting prior sexual intercourse (oral, anal or vaginal) in the age groups 15-19 years and 20-19 years had decreased in the later time period. A British study that investigated the seroprevalence of HSV-2 in a sexually transmitted diseases clinic found that homosexual men had a significantly increased risk of being HSV-2 positive than heterosexual men, although the results were not stratified by numbers of sexual partners (Cowan *et al*, 1994).

1.7.2 Cytomegalovirus

Like EBV, CMV may be transmitted through salivary contact. CMV is also found in semen and cervical secretions with sexual transmission thought to be a significant route of person-to-person spread. In addition transmission occurs transplacentally, during birth, via breast

milk, and iatrogenically, by blood transfusion or organ transplantation (Britt, 2005; Ho, 1990; Pass, 2001). The seroprevalence of CMV infection increases with age although the age at initial infection varies with living conditions, occurring earlier in developing countries and in less affluent communities of the developed world. In the USA and Europe rates of CMV infection are higher in non-white ethnic groups, although this appears to be a result of socio-economic differences, rather than any true difference in susceptibility (Pass, 2001). Although early acquisition of CMV is generally related to poor socio-economic conditions, Scandinavia and Japan are notable exceptions, because of high rates of perinatal transmission and group day care of infants (Dollard *et al*, 2007; Ho, 1990; Pass & Hutto, 1986). Amongst CMV positive mothers the detection of CMV DNA in breast milk is almost universal (Hamprecht *et al*, 2001), however, the prevalence of CMV varies as does the acceptance of breast feeding (Pass & Hutto, 1986) resulting in variations in perinatal acquisition. In the UK and USA where fewer mothers have had CMV infection and breast feeding is less consistently practised; only 10% of one year olds have acquired CMV as opposed to a seroprevalence of 39-56% in infants in Finland, and Japan (Stagno *et al*, 1980). Group care of young children appears to facilitate CMV transmission with 80% of children acquiring CMV in this setting compared with 20% of children from a similar socio-economic background cared for at home (Pass & Hutto, 1986). Later in life the prevalence of CMV infection is affected by sexual behaviour with positive correlations noted with increasing duration of sexual activity in years, numbers of lifetime partners, and the presence of sexually transmitted infections (Chandler *et al*, 1985a; Chandler *et al*, 1985b; Collier *et al*, 1990; Sohn *et al*, 1991; Stanberry *et al*, 2004). The use of barrier contraception may protect against the acquisition of CMV (Chandler *et al*, 1985a; Collier *et al*, 1990) although after regression analysis Chandler *et al* found only a trend ($p=0.06$) towards significance.
Dan and Chang (1990) found the likelihood of CMV seropositivity was positively correlated with EBV seropositivity in Chinese students in Hong Kong, ($p=0.02$), although fewer students had acquired CMV (60%) than EBV (94%) (see Table 3).

Table 3 Proportions of students with antibodies to EBV and CMV at The Chinese University of Hong Kong

†	EBV-	EBV+	Totals
CMV+	6 (2.1%)	167 (57.6%)	173 (59.7%)
CMV-	12 (4.1%)	105 (36.2%)	117 (40.3%)
Totals	18 (6.2%)	272 (93.8%)	290

† Data from Dan and Chang (1990)

A British study of EBV in colleges and universities showed that CMV seroprevalence was 30% at college entry and acquisition was less than that of EBV at 1.4% in seven months, considerably lower than the 12% acquisition rate of EBV during the same period (Anon., 1971). A similar seroprevalence of 29% was found in 199 first year medical students at St. Thomas's Hospital, London in the late 1980s (Best *et al*, 1994).

1.8 EBV Genome

In the infectious virion the DNA is double-stranded and linear, whilst in infected cells the genome persists as an extra-chromosomal circular episome. This circularisation is mediated by a terminal repeat sequence at each end of the linear molecule. The exact number of the 500 base pair (bp) repeat sequences is variable, a feature that has been used to assess clonality of the virus in tumours (Raab-Traub & Flynn, 1986). Within the genome there is another larger repeat sequence known as the major internal repeat or IR1, which comprises 5-10 copies of a 3072 bp sequence. It contains the Wp promoter for the EBNA genes which is active when the virus first infects a B lymphocyte. The genes assembled on either side of IR1 are known as the unique short region (U_S) and unique long region (U_L). The U_L is further subdivided by an additional three internal repeats. Overall the EBV genome has coding potential for about 70 proteins of which the latent genes (see 1.8.1 and Table 4) allow the establishment of a latent infection with limited viral gene expression but without the production of virions. The EBV genome was sequenced using DNA fragments obtained by *Bam*H1 restriction enzyme digestion (Baer *et al*, 1984). The *Bam*H1 restriction fragments are named according to their size, A being the largest. Genes are designated according to the *Bam*H1 fragment in which they are located e.g. the transcription factor BZLF1 is located in the first (1) leftward (L) open reading frame of the *Bam*H1 (B) fragment (Z) (Baer *et al*, 1984).

1.8.1 Latent Gene Expression

EBV possesses genes to facilitate viral survival in the host in order to achieve the state of

latency. These are the EBV nuclear antigen genes (EBNA) 1, 2, 3A, 3B and 3C, leader protein (LP), the latent membrane protein genes LMP1, 2A and 2B, EBV encoded RNAs EBER 1 and 2, and the complementary strand transcripts (CSTs) also known as *Bam*H1 A rightward transcripts (BARTs). In EBV-infected cells lytic infection resulting in cell destruction is unusual in vitro; instead a latent infection is established in which the episomal viral DNA is replicated and transmitted to the daughter cells. The expression of the latent genes is tightly regulated by methylation of the gene promoters (Tao & Robertson, 2003) in order to allow EBV to persist within the host without eradication by the host immune response. Recently several EBV-encoded microRNAs have been described (Pfeffer *et al*, 2004). Clustered in two regions of the genome (BHRF1 and BART) these RNAs are thought to function as host and viral gene regulators, which, unlike proteins, do not elicit an immune response. Several patterns of expression of the latent genes in EBV associated tumour cells are recognised as shown in the Table 4 below.

Table 4 The expression and function of EBV latent genes

Latent Gene	Alternative designation	Function	Required for immortalisation	PBM[†]	IM[‡]	PTLD*	BL	NPC	HD	T cell lymphoma	Gastric ca
EBNA1		Maintains EBV genome as an episome during cell division Contributes to transcriptional regulation	+	+	+	+	+	+	+	+	+
EBNA2		Transcriptional activator of cellular and viral genes LMP1 and LMP2A and B and the Cp promoter	+	-	+	+	-	-	-	-	-
EBNA3A-C	EBNA3,4,6	Transcriptional regulators Affects activation functions of EBNA2 3C upregulates LMP1 promoter	3A and 3C +	-	+	+	-	-	-	-	-
EBNA LP	EBNA5	Enhances the immortalising activities of EBNA2 co-activates EBNA2 responsive genes	-	-	+	+	-	-	-	-	-
LMP1		Mimics CD40 giving growth and differentiation signals to B-cells Protects B-cells against apoptosis	+	-	+	+	-	+/-	+	+	-$

LMP2A	TP-1	LMP2A Mimics signalling through the B-cell receptor allowing proliferation and survival of B-cells Blocks reactivation of latently infected cells into the lytic cycle by blocking signalling through the B-cell antigen receptor complex	-	+†	+	+	+(-)	+	+	+	+
LMP2B	TP-2	Unknown, may regulate LMP 2A	-				-(+)	+	+	+	
EBERs 1&2 Non-polyadenylated RNAs		Possible role in RNA splicing Block effects of IFN-γ by inhibiting expression of interferon inducible genes Possible oncogenic role by upregulating Bcl-2 expression	-	+	+	+	+	+	+	+	+
*Bam*HI-A rightward transcripts (BARTs, also known as CSTs)		Unknown	-	+	+	+	+	+	+	+	+

EBNA Epstein-Barr nuclear antigen; LP leader protein; LMP latent membrane protein; TP terminal protein; EBER Epstein-Barr encoded RNA; CST complementary strand transcripts; BARTs *Bam*HI-A rightward transcripts; PBM peripheral blood mononuclear cells; IM infectious mononucleosis; PTLD post transplant lymphoproliferative disease; BL Burkitt's lymphoma; NPC nasopharyngeal carcinoma; HD Hodgkin's Disease; ca carcinoma; IFN interferon

† extent of gene expression debated (Hochberg *et al*, 2004; Qu & Rowe, 1992; Tierney *et al*, 1994)

‡ in IM tonsil and lymph node gene expression varies, EBNA2+LMP1- reported in addition to latency 1, 2, & 3 (Kurth *et al*, 2000; Niedobitek *et al*, 1997)

$ occasional cells positive for LMP1 reported (Osato & Imai, 1996)

* PTLD with more restricted gene expression also described (Brink *et al*, 1997; Rea *et al*, 1994)

Adapted from (Epstein & Crawford, 2005; Kieff & Rickinson, 2007; Young *et al*, 2000; Young & Rickinson, 2004)

Latency type three, as seen in post-transplant lymphoproliferative disease (PTLD), involves expression of all nine of the latent genes, the non-polyadenylated EBERs 1 and 2, and the *Bam*H1 A transcripts. Type two latency is typified by Hodgkin's disease, nasopharyngeal carcinoma and T-cell lymphomas, and shows transcription of EBNA1, LMP1 and 2, EBERs and *Bam*H1 A transcripts. BL shows the latency type one pattern with expression of EBNA1, EBERs and *Bam*H1 A transcripts only. The form of latency found in latently infected resting memory B-cells is debated. EBNA1 is expressed during cell division (Hochberg *et al*, 2004; Yates *et al*, 1985), and there may also be limited transcription of LMP2A, which prevents cells from entering the lytic cycle, although the extent of this is contested (Hochberg *et al*, 2004; Qu & Rowe, 1992; Tierney *et al*, 1994).

1.8.2 EBV Types 1 and 2

Two types of antigenically distinct EBV exist which differ in the coding regions of EBNA 2, 3A, 3B, 3C and LP (Farrell, 2005; Sample *et al*, 1990). The type 1 and 2 EBNA2 proteins share only 56% of amino acids and have differing functional capabilities with type 2 being less capable of transforming B lymphocytes (Apolloni & Sculley, 1994; Rickinson *et al*, 1987). The two types are not distributed equally with type 2 strains being more common in Africa and Papua New Guinea (Young *et al*, 1987; Zimber *et al*, 1986). Subsequent PCR based studies suggested that type 2 strains and simultaneous infections with both types were more widespread than previously thought (Apolloni & Sculley, 1994; Sixbey *et al*, 1989). Infection with type 2 has been associated with high risk sexual behaviour in heterosexual and homosexual men in both HIV-infected and uninfected individuals (van Baarle *et al*, 2000). In contrast, HIV-infected haemophiliac males have a prevalence of type 2 infection that is closer to that of the general population (Yao *et al*, 1998). Amongst HIV seropositive populations type 2 EBV is over represented in the EBV-malignancies that arise in the context of HIV related immunosuppression (Powles & Bower, 2000).

1.9 Life Cycle of EBV

1.9.1 B-Cell Biology

A brief outline of normal B lymphocyte development is helpful in understanding the lifecycle of EBV as the two are inextricably linked. Naïve B-cells (denoted by bearing IgD and lacking CD27) released from the bone marrow have not responded to antigen, although, due to the variation in assortment of the immunoglobulin genes, each is already programmed to bind a specific antigen, known as their cognate antigen, via their B cell receptor (BCR). The B-cells circulate in the periphery and secondary lymphoid organs, although cells arising

in the tonsil and adenoids appear to preferentially traffic back to the lymphoid tissue of Waldeyer's ring (Laichalk *et al*, 2002). When naïve B-cells meet an antigen which attaches to their BCR they become activated and home to the T-cell zone of lymphoid organs. If they interact with dendritic cells and T-helper cells they will either differentiate into short-lived plasma cells, producing low affinity, predominantly IgM antibody, or, migrate into B cell follicles and start to proliferate and differentiate into centroblasts, in what becomes a germinal center. Centroblasts undergo somatic hypermutation in the variable region of their immunoglobulin genes which produces cells with variants of their original BCR, some of which will have a higher affinity for the antigen. Class switching of the immunoglobulin heavy chain occurs under the influence of cytokines and CD40 ligand (McHeyzer-Williams & McHeyzer-Williams, 2005). The centroblasts differentiate into resting centrocytes and those with a BCR which has a high affinity are selected to avoid apoptosis, by receiving survival signals from germinal center T-cells and dendritic cells. These include interaction of the BCR with cognate antigen on dendritic cells and ligation of CD40 on the B-cell with CD40 ligand on T-cells. Finally the B-cells exit the germinal center as antigen-specific $CD27^+$ memory cells or long-lived plasma cells for a life of antigen surveillance in peripheral blood and secondary lymphoid organs. Following re-challenge with their cognate antigen they differentiate into plasma cells and secrete antibody.

1.9.2 *In vitro* Study of EBV Infection

EBV attaches to the receptor for the complement component C3d, also known as CD21, on B-cells via its glycoprotein (gp) 350/220, an analogue of gC (Fingeroth *et al*, 1984). Thereafter gp42 (an analogue of gD, but without homology) attaches to HLA class II at specific amino acid sites, which triggers fusion of the virus envelope with the cell membrane via gp110 (gB), gp85 (gH) and gp25 (gL) (Spear & Longnecker, 2003). Epithelial cells lack HLA class II and generally CD21 is also absent, and in these cells binding of EBV is mediated by gp85 and gp25 to an as yet unidentified cellular receptor , and by another virus glycoprotein, BMRF2, to the cellular $\alpha5\beta1$ integrin (Hutt-Fletcher, 2005; Molesworth *et al*, 2000; Tugizov *et al*, 2003). Virus derived from B-cells more readily infects epithelial cells than B-cells due to the trapping of gp42 by HLA II as EBV leaves the cell (Borza & Hutt-Fletcher, 2002), whereas EBV derived from epithelial cells more readily infected B-cells, stimulating the debate on cell type initially infected in natural infection. Opinion is divided as to whether infection of the epithelial occurs prior to infection of B-cells, or whether infection of B-cells occurs directly via the large surface area of the tonsillar crypts where a specialised discontinuous epithelium exists (Howie, 1980; Perry & Whyte, 1998; Perry *et al*,

1988). Tugizov *et al* (2003) demonstrated three modes of EBV infection in their in vitro cell cultures of polarized tongue and nasopharyngeal epithelial cells : 1) direct cell to cell contact at the apical cell membrane with EBV-infected lymphocytes; 2) entry at the baso-lateral membranes of cell-free virions; and 3) virus spread across lateral membranes to adjacent epithelial cells.

Early work found EBV-infected epithelial cells in IM (Lemon *et al*, 1977; Sixbey *et al*, 1984), a finding refuted by later studies (Anagnostopoulos *et al*, 1995; Karajannis *et al*, 1997; Niedobitek *et al*, 2000), which may reflect differences in methodology. It is possible that a transient infection of epithelial cells occurs as prelude to infection of B-cells; this would occur prior to the onset of IM symptoms and could therefore be missed in studies involving tonsils excised in acute IM.

1.9.3 A Model of EBV Persistence

The hypothesis espoused by Thorley-Lawson is that EBV uses the normal processes of B-cell maturation and development to persist in the host, without causing disease, and escapes elimination by the immune response through maintaining latency in memory cells without expressing any viral proteins (Hochberg *et al*, 2004; Thorley-Lawson, 2005).

In this hypothesis EBV infects naive B-lymphocytes in the lymphoid tissue of Waldeyer's ring where lytic replication takes place as well as proliferation of the infected B-cells with expression of all latent genes. The EBV activated B-cells migrate into follicles to form a germinal centre where EBNA2 gene expression is switched off (Thorley-Lawson, 2005). EBV uses expression of LMP1 (a functional homologue of CD40) and LMP2A, which is thought to replace the survival signal normally received from the BCR, to supplant the normal cell survival signals required in the germinal centre (Caldwell *et al*, 1998; Gires *et al*, 1997; Kilger *et al*, 1998; Uchida *et al*, 1999; Zimber-Strobl *et al*, 1996). The infected B-cells survive and differentiate to be released to the periphery as resting memory cells IgD-$CD27^{+}$. Once in the peripheral blood these resting memory cells divide only infrequently, expressing EBNA1 when they do so, in order to maintain the viral episome (Hochberg *et al*, 2004; Yates *et al*, 1985). Otherwise EBV viral gene expression is minimal; several groups have detected RNA transcripts of LMP2A (Babcock *et al*, 1999; Miyashita *et al*, 1997; Qu & Rowe, 1992; Tierney *et al*, 1994) and some have also detected transcripts of EBNA1 (Miyashita *et al*, 1997; Tierney *et al*, 1994). Single cell analysis by Thorley-Lawson (Hochberg *et al*, 2004; Thorley-Lawson, 2005) demonstrated that most memory cells do not express any EBV latent genes. The memory B-cells are thought to cycle between the periphery and Waldeyer's ring (Laichalk *et al*, 2002) and are stimulated into lytic replication by differentiating into plasma cells (Crawford & Ando, 1986; Laichalk & Thorley-Lawson,

2005). The signals causing the differentiation into plasma cells are unclear and may be either the recognition of their cognate antigen, when cross-linking of their BCR occurs, or an antigen independent process, involving bystander T-cell help, similar to that involved in B-cell memory (Laichalk & Thorley-Lawson, 2005). This allows the production of infectious virus, in the oropharynx, which may be transmitted to new susceptible hosts. Anagnostopoulos *et al* (1995) propose that inter-individual transmission of EBV occurs via the transfer of EBV-infected lymphocytes based on their in situ hybridisation study of tonsils removed during IM.

1.9.4 Transmission

Studies of the epidemiology of IM had failed to show any increased risk of IM amongst susceptible roommates of cases (Hallee *et al*, 1974; Sawyer *et al*, 1971). Hoagland had retrospectively found a history of intimate oral contact with exchange of saliva amongst many cases (Hoagland, 1964; Hoagland, 1955). A study of 45 EBV seronegative women, who were seen every 6 months in a British family planning clinic, reported a median time to seroconversion of 25 months (range 1-60 months) (Woodman *et al*, 2005). The authors reported that "acquisition of infection is rarely contemporaneous with the first exposure to an infected partner and that considerable time may have to elapse before a woman becomes infected."

1.9.5 Oral Secretions

Cell free virus, present in throat washings of seropositive individuals may be detected by the immortalisation of cultured B lymphocytes obtained from a seronegative donor. This technique, utilised in early studies, demonstrates the presence of EBV in the throat gargles of 14-25% of healthy asymptomatic adults (Chang *et al*, 1973; Chang *et al*, 1978; Ferbas *et al*, 1992; Gerber *et al*, 1972; Haque & Crawford, 1997). Longitudinal study of EBV-infected individuals showed that the excretion of cell free virus in throat gargles was present on every occasion in 25% and intermittently detected in a further 66%, with only 2/24 (8%) not having detectable EBV on any occasion (Yao *et al*, 1985). In acute IM 50% of patients had cell free virus in their throat gargles using the same technique (Gerber *et al*, 1972). Miller *et al* found EBV in 92% of IM cases again by the technique of B-cell transformation but their sample processing method involved cell lysis, rather than using a cell free filtrate (Miller *et al*, 1973). The increased rate of EBV detection after IM persisted for more than three months following diagnosis (Miller *et al*, 1973; Niederman *et al*, 1976). Throat wash samples obtained from immunosuppressed patients show rates of B-cell immortalisation of 38%-100% (Chang *et al*, 1978; Ferbas *et al*, 1992; Strauch *et al*, 1974). The more sensitive

technique of PCR has demonstrated EBV DNA in the throat wash samples of 48-62% asymptomatic individuals (Conacher *et al*, 2005; Haque & Crawford, 1997) and 97% of IM cases (Balfour *et al*, 2005).

1.9.6 Genital Secretions

The possibility of sexual transmission is raised by the finding of cell free virus from cervical washings in 4/28 (14%) women, and by positive PCR in 28-40% of cervical samples in four other studies (Bevan *et al*, 1989; Näher *et al*, 1992; Sixbey *et al*, 1986; Taylor *et al*, 1994; Voog *et al*, 1995). Ten of 21 (48%) symptomatic males attending a sexually transmitted diseases clinic had EBV detected by PCR in cell-free filtrates of their urethral discharge (Israele *et al*, 1991). One case report describes the detection of EBV in genital ulcers in a female with IM (Portnoy *et al*, 1984). Additional reports record the temporal association of genital ulceration and IM, mostly in female patients, although not all had been sexually active (Hudson & Perlman, 1998; Lawee & Shafir, 1983; Lorenzo & Robertson, 2005; Wilson, 1993).

1.9.7 Breast Milk

Like CMV, EBV has been detected in breast milk. Junker *et al* detected EBV DNA in 60 of 132 (46%) of samples donated to a Canadian breast milk bank (Junker *et al*, 1991). It is not clear whether breast milk is a source of EBV infection in infants; the transplacental transmission of maternal antibodies to EBV in term infants would appear to protect against early infection as evidenced by the abrupt onset of seroconversions after 8 months of age (Chan *et al*, 2001).

1.9.8 Iatrogenic Acquisition of EBV

EBV may be acquired as a consequence of blood transfusion (Alfieri *et al*, 1996; Gerber *et al*, 1969) or organ transplantation (Cen *et al*, 1991; Haque *et al*, 1996).

1.10 The Immune Response to EBV

EBV, a potentially oncogenic virus, elicits a broad array of responses from the immune system and whilst these on the one hand might be regarded as ineffective, as the virus is not eradicated, on the other the virus is sufficiently controlled in the vast majority of healthy individuals so that no disease state ensues. The virus has evolved a number of immune evasion mechanisms which allow persistence during the lifetime of the host and the opportunity for transmission to new susceptibles.

Much of the data regarding the immune response to primary EBV infection are gathered from patients diagnosed with IM as this is a detectable event. IM probably represents an

exaggerated response to the virus, and subclinical infection is likely to elicit a less marked cellular immune response.

1.10.1 Innate Immune Response

Natural killer cells form part of the innate response, express the surface marker CD56, show cytotoxic activity – especially after activation by interferon (IFN)-α or β – and may themselves produce IFN-γ, tumour necrosis factor (TNF) and chemokines (Biron & Brossay, 2001). In IM natural killer (NK) cells form a proportion of the population of atypical lymphocytes (Tomkinson *et al*, 1987). NK cells are thought to contribute to the control of EBV, as evidenced by the reports of severe EBV infection in persons with NK cell defects (Joncas *et al*, 1989; Merino *et al*, 1983). Boys with X-linked lymphoproliferative disease have a defective or absent intracellular protein known as SAP (signalling lymphocytic activation molecule [SLAM] associated protein). In health SAP modulates the function of many immune cells, including NK cells, by receiving signals from surface receptors. It is thought that defective signalling, as a consequence of altered SAP, results in failure of activation of NK cells which are then unable to kill EBV-infected B-cells (Nichols *et al*, 2005; Parolini *et al*, 2000).

Gamma-delta T-cells form 2-7% of peripheral blood T-cells (Borst *et al*, 1988; Hassan *et al*, 1991; Lanier & Weiss, 1986) and have T-cell receptors composed of γ and δ chain heterodimers. They differ from conventional αβ-T-cells in that they do not require antigen processing, respond to non-peptide antigens and may function as antigen presenting cells (Brandes *et al*, 2005; Chen & Letvin, 2003). Gamma-delta T-cells proliferate following recognition of their non-peptide antigen and show a rapid and prolonged response following re-exposure to the same antigen thus appearing to participate in both innate and adaptive immune responses. Gamma-delta cells are thought to exert cytotoxicity by both perforin mediated lysis and Fas-mediated apoptosis, and also to be a source of cytokines including IFN-γ and TNF-α (Kabelitz, 1999). Marked increases in γδ T-cells have been noted in patients with certain infectious diseases, particularly intracellular pathogens (Bertotto *et al*, 1993; Ito *et al*, 1992; Jouen-Beades *et al*, 1997; Schneider *et al*, 1997), including EBV (De Paoli *et al*, 1990; Hassan *et al*, 1991).

1.10.2 Adaptive Cell Mediated Immune Response

During IM the most obvious feature of the immune response to EBV is the massive lymphocytic response, observable on a blood film as atypical lymphocytes. Early studies determined that these were mainly $CD8^+$ T-cells with contributions of $CD4^+$ T-cells and NK cells (Crawford *et al*, 1981; Tomkinson *et al*, 1987). $CD8^+$ T-cells recognise the antigen

specific for their receptor, and may either kill the infected cell directly, by osmotic lysis or Fas-mediated apoptosis, or, release soluble antiviral factors, including IFN-γ and TNF-α. Some have argued that this exaggerated cytotoxic T-cell response is induced by a superantigen triggering non-specific T-cell activation and proliferation (Sutkowski *et al*, 2001; Sutkowski *et al*, 1996), however, analysis of the T-cell receptors shows an oligoclonal or clonal response in primary EBV infection (Callan *et al*, 1996). More recently the use of HLA-tetramer technology has allowed more detailed dissection of the $CD8^+$ T-cell response, which is surprisingly large with up to 44% of all $CD8^+$ cells in a single individual being specific for one EBV epitope (Callan *et al*, 1998). The exact epitopes eliciting the $CD8^+$ response varies with the HLA type of the individual. Whilst $CD8^+$ expansions to both lytic and latent antigens is seen, the response to the lytic antigens occurs earlier and is of a greater magnitude (Callan, 2004; Catalina *et al*, 2001; Hislop *et al*, 2002). The response to the lytic antigens is predominantly directly towards immediate early and early gene products rather than the late structural proteins (Pudney *et al*, 2005; Steven *et al*, 1997). In the response to EBV latent antigens a distinct hierarchy of EBV antigens is observed; epitopes derived from EBNA 3A, 3C and 3B are the most prevalent followed by LMP2. $CD8^+$ cells specific for EBNA2, LP and LMP1 epitopes are less commonly detected (Rickinson & Kieff, 2007). For many years it was thought that the EBNA1 protein did not elicit a cytotoxic $CD8^+$ response as such cells were not detected in the T-cells responding to EBV-infected B lymphoblastoid cells (Rickinson and Kieff, 2007). Furthermore this protein contained a glycine-alanine repeat domain which could not be processed by the proteosome (Levitskaya *et al*, 1997); the main method by which epitopes are generated for presentation to cytotoxic lymphocytes on class I HLA molecules. More recently this sequence has also been shown to reduce its own translation from EBNA1 mRNA (Yin *et al*, 2003) further reducing EBNA1 epitope presentation. In spite of these unusual evasion mechanisms EBNA1 is a frequent $CD4^+$ T-cell target (Leen *et al*, 2001; Münz *et al*, 2000) and now using the more sensitive technique of IFN-γ detection, EBNA1-specific $CD8^+$ T-cells have been detected (Münz, 2004).
T-cells are also critical in the control of latent EBV infection as evidenced by the rising numbers of infected B-cells that occur as immunity declines in advanced HIV (Birx *et al*, 1986) and following organ transplantation (Lucas *et al*, 1996; Savoie *et al*, 1994). Furthermore, EBV-associated B-cell lymphomas arising in settings of immunodeficiency, such as organ transplantation (Armitage *et al*, 1991; Ho *et al*, 1988) may be successfully treated with infusions of EBV-specific T-cells (Haque *et al*, 2002; Rooney *et al*, 1998). Irrespective of whether the acute response in IM is exaggerated or not, work examining the frequencies of EBV-specific $CD8^+$ cells in healthy volunteers has demonstrated on-going

high levels of cells specific for both lytic and latent antigens which can be as prevalent as 5.5% for a single epitope, even in persons without preceding IM (Tan *et al*, 1999).
The final composition in the epitope specificity of $CD8^+$ and $CD4^+$ cells following IM and asymptomatic seroconversion has not been formally compared but is thought to result in similar outcomes (Callan, 2004).
The contribution of the $CD4^+$ response to the control of EBV infection is less well defined but is the subject of on-going study. In IM, the proliferation of $CD4^+$ cells is much less than that of $CD8^+$ cells (Macallan *et al*, 2003), however study of other infections including murine lymphocytic choriomeningitis (LCMV) infection suggests that they are likely to be crucial in the mounting of an effective immune response (Zajac *et al*, 1998). In HIV infection, secretion of IFN-γ by EBV-specific $CD8^+$ cells was noted to fall with decreasing CD4 counts (van Baarle *et al*, 2001). After the institution of highly active antiretroviral therapy detection of IFN-γ increased (Kostense *et al*, 2002). At the time of diagnosis of IM EBV–specific $CD4^+$ cells directed against both lytic antigens and the latent antigen EBNA3A are present (Precopio *et al*, 2003). Recent in vitro studies have concentrated on EBNA1-specific $CD4^+$ cells which may be critical in the defence against EBV-associated malignancies (Münz, 2004). Using overlapping peptides to stimulate IFN-γ release, CD4 epitopes within the latent proteins EBNA3C, LMP1 and LMP2 have also been identified, although $CD4^+$ cells directed against LMP1 and 2 occur in fewer individuals than those to EBNA1 or EBNA3C (Leen *et al*, 2001).

1.10.3 Adaptive Humoral Immune Response

B-cells are the site of latent infection and are also responsible for the humoral response to the virus, however, the role of antibody in ameliorating primary infection and in preventing subsequent re-infections is unknown. The early antibody response in IM is characterised by IgM and IgG to "viral capsid antigen"; actually a mixture of antigens made late in lytic replication including the nucleocapsid and gp110, and early lytic cycle antigens so-called "early antigen" (Rickinson & Kieff, 2001). Neutralising antibody initially of the IgM class is produced against the major envelope glycoprotein gp350 with IgG to this antigen rising relatively late (Rickinson & Kieff, 2007; Thorley-Lawson & Poodry, 1982). There is a dichotomous antibody response to the nuclear antigens, with those against EBNA2 and 3 being formed early, and those against EBNA1 being delayed (Henle *et al*, 1987). In the acute phase of IM a virus-induced polyclonal activation of B-cells occurs and a range of "heterophile" and autoantibodies (mostly of the IgM class) are produced (Henle & Henle, 1979). Heterophile antibodies that are able to agglutinate horse and sheep erythrocytes form

the basis of the "monospot" and Paul-Bunnell diagnostic tests used in acute IM (Davidsohn, 1937; Lee *et al*, 1968a; Paul & Bunnell, 1932) (see laboratory findings 2.1.1).

1.11 Immune Evasion

In order to maintain life-long persistence of EBV without eradication a number of evasion strategies have evolved. The most important is the ability to remain latent in memory B-cells with little or no gene expression as described above (see 1.9.3). EBNA1 is an unusual protein with a glycine-alanine repeat domain, which reduces its own transcription and proteosomal degradation as discussed above (see 1.10.2). These factors do not make EBNA1 invisible to the immune system but are thought to reduce antigen presentation via class 1 MHC molecules on infected B-cells. EBV has a number of homologues of human genes which are thought to alter the immune response. Viral IL-10 has 84% homology with the human gene; a cytokine with diverse anti-inflammatory and inhibitory effects on T-cells, monocytes, macrophages and NK cells and which also stimulates the growth and differentiation of B-cells (Cohen, 1997; Moore *et al*, 2001). EBV also subverts the effects of class I and II interferons by: synthesising viral receptors which compete with the intended IFN receptor; generating interfering RNAs which inhibit interferon-directed protein synthesis; and down-regulating IFN-induced transcription (Cohen & Lekstrom, 1999; Morrison *et al*, 2001; Sharp *et al*, 1999; Tortorella *et al*, 2000). The survival of EBV-infected cells is promoted by several anti-apoptotic mechanisms including the production of two homologues of the human gene Bcl-2, BHRF1 and BALF1; and the up-regulation of bcl-2 itself by the viral gene LMP1 (Henderson *et al*, 1991; Henderson *et al*, 1993; Marshall *et al*, 1999). Ressing *et al* have demonstrated that lytically infected cells secrete soluble gp42, a glycoprotein that binds HLA class II molecules, which may help to protect EBV-infected cells from $CD4^+$ T-cell recognition (Ressing *et al*, 2005).

2 Disease Burden

Infection with EBV is a significant public health concern. It is the known aetiological agent of infectious mononucleosis, oral hairy leukoplakia and the majority of cases of B-cell lymphoproliferative disease. Furthermore EBV is thought to have a direct role in the pathogenesis of nasopharyngeal cancer, endemic Burkitt's lymphoma, around 40% of Hodgkin's disease and 10% of gastric carcinoma, the fourth most prevalent cancer worldwide. It is also associated with a number of less common tumours, and has a putative role in several other tumours and immune mediated diseases. Understanding of the host

immune response, the strategies evolved by the virus to evade immune control and of the stages involved in oncogenesis are important in devising vaccines, therapies, and public health measures to reduce the disease burden.

2.1 Diseases of Lymphocyte Origin

2.1.1 Infectious Mononucleosis

Clinical features and complications

After an incubation period of between four and seven weeks the symptoms of IM typified by fever, sore throat and lymphadenopathy may arise (Hoagland, 1964; Hoagland, 1960; Johannsen *et al*, 2005). The illness may be subacute in presentation resulting in difficulty in pin-pointing the exact day of onset (Hoagland, 1960). Not all cases have pronounced sore throat or lymphadenopathy; in some fever may predominate, whilst occasionally in others, the presentation may be with an unusual complication.

Pharyngitis is evident in 85% and exudates may be seen in 22%. Lymphadenopathy particularly affecting the posterior triangle of the neck is present in most cases; axillary or groin nodes are detected in around half of cases (McCarthy & Hoagland, 1964). Splenomegaly is clinically detectable in up to 17%; an ultrasound study demonstrated splenic enlargement in all of 29 cases (Dommerby *et al*, 1986). Hepatomegaly is clinically detectable in 10-15% of cases and in 50% by ultrasound (Dommerby *et al*, 1986; Johannsen *et al*, 2005). Other features include petechiae at the junction of the soft and hard palate, and eyelid oedema (McCarthy & Hoagland, 1964). Rashes, including macular erythema, petechiae and urticaria are reported in 3-16% of cases, however, the concurrent administration of ampicillin results in a rash in around 90% or more (Grotto *et al*, 2003; McCarthy & Hoagland, 1964; Pullen *et al*, 1967), and calculated from Kerns *et al* (Kerns *et al*, 1973). The acute features resolve over 3-4 weeks (Chretien *et al*, 1977), however thereafter a fatigue state lasting a median of 8 weeks is common (White *et al*, 1998) and occasionally depression may follow (Cadie *et al*, 1976; Petersen *et al*, 2006; White *et al*, 1998). A number of complications are well recognised (see Table 5) particularly hepatitis, jaundice, thrombocytopenia, haemolytic anaemia, splenic rupture, and fatigue. Rarer complications include encephalitis, demyelination, arrhythmia, pancreatitis, gastrointestinal haemorrhage, interstitial nephritis and pulmonary infiltrates (Hickey & Strasburger, 1997; Johannsen *et al*, 2005). Death as a result of IM is very rare and may be the result of an underlying immunological defect, particularly the X-linked Duncan Syndrome (Bar *et al*, 1974; Mroczek *et al*, 1987; Purtilo *et al*, 1975). Other underlying defects resulting in severe disease are recognised, including defective NK cell function (Joncas *et al*, 1989), perforin

gene mutations (Katano *et al*, 2004) and selective IFN-γ deficiency (Andersson *et al*, 1999). Even in those without immune defects, fatal outcomes may sometimes occur as a result of airway obstruction, neurological complications, hepatic failure, splenic rupture, myocarditis, cardiac arrhythmia, secondary bacterial infection or haemorrhage (Andersson, 1991; Carrington & Hall, 1986; Farley *et al*, 1992; Hickey & Strasburger, 1997; Johannsen *et al*, 2005; Kennard & Swash, 1981; Lukes & Cox, 1958; Markin *et al*, 1987; Penman, 1970).

Organ/System	Complication
Liver	Clinical jaundice 5%, fulminant hepatitis (rare)
Skin	Rashes 3-16%; following the administration of ampicillin 86-98%
Haematological	Thrombocytopenia, haemolytic anaemia, neutropenia
Psychiatric	Depression, anxiety
Immunological	Secondary infection, streptococcal sore throat, sepsis in association with neutropenia, mesenteric adenitis, depressed cell mediated immunity
Spleen	Splenic rupture 0.1-0.5% spontaneous or after mild trauma (usually in males), splenic infarction
Neurological	Encephalitis, acute cerebellar syndrome, aseptic meningitis, Guillain Barré Syndrome, cranial nerve palsy especially VII, transverse myelitis, seizures, mononeuritis, optic neuritis, cerebral haemorrhage
Gastrointestinal	Haemorrhage secondary to mucosal erosion, pancreatitis
Genitourinary	Haematuria, interstitial nephritis, glomerulonephritis (rare), genital ulceration
Cardiac	Myocarditis, pericarditis, arrhythmia, electrocardiogram changes
Respiratory	Respiratory tract obstruction, interstitial pneumonitis (rare)

Table 5 Complications of acute infectious mononucleosis

Laboratory findings

Prior to the availability of specific serological tests and the recognition of heterophile antibodies IM was known to be associated with an absolute lymphocytosis and atypical lymphocytes (Downey & McKinlay, 1923; Sprunt & Evans, 1920). The early studies relied on strict adherence of the following diagnostic criteria 1) absolute lymphocytosis ≥ 4500 /μl, 2) relative lymphocytosis >50% and 3) atypical lymphocytes >10%, but after the development of specific serological tests, and in particular anti-VCA (viral capsid antigen)

IgM, it was recognised that approximately 60% of acute IM cases would be excluded by these early criteria if only one blood count observation was performed (Fleisher *et al*, 1983). Thus an absolute lymphocytosis is typical but not always present, lymphopenia is rarely present and atypical lymphocytes are usually present, but often not at the originally described level of 10%. Mild thrombocytopenia is common and neutropenia less so (Carter, 1965; Carter, 1966a).

Prior to the discovery of EBV as the aetiological agent in IM the production of antibodies to animal antigens, particularly agglutinins to sheep erythrocytes, recognised in 1932 by Paul and Bunnell, was used for laboratory confirmation of IM. Davidsohn refined the Paul-Bunnell test with the use of guinea pig kidney antigen, which improved specificity, by removing Forssman antibodies (animal erythrocyte agglutinins in human serum not related to acute EBV infection) (Davidsohn, 1937).

Elevations of one or more liver function tests bilirubin, alkaline phosphatase, aminotransferases occur in around 80% of IM cases, whilst clinical jaundice is apparent in up to 5% of patients (Finkel *et al*, 1964; Paublini *et al*, 1977). Hyperbilirubinaemia may result from impaired hepatic clearance or increased red blood cell (RBC) destruction as a result of haemolysis or a combination of these. Jaundice is more common in older patients and is a frequent accompaniment of patients that succumb to fulminant infection (Horwitz *et al*, 1983; Markin *et al*, 1987).

Pathogenesis of IM

The exaggerated cytotoxic lymphocyte response and cytokine secretion are thought to account for the symptoms of IM but the reasons for the development of the exaggerated immune response are not known (Attarbaschi *et al*, 2003; Foss *et al*, 1994; Linde *et al*, 1992). Why some individuals develop IM on primary EBV infection and others do not is unexplained, but both host and viral factors may be important. Small studies of HLA types and the occurrence of IM have not detected any association (Rosdahl & Svejgaard, 1979; Schiller & Davey, 1974; Ythier *et al*, 1983). In nasopharyngeal carcinoma an association has been detected between HLA type and the occurrence of this EBV-related tumour within families but the HLA type differs between affected family groups (de-Thé, 2005); an analogous situation may exist in IM. Genetic polymorphism of the IL-10 gene promoter has been associated with variation in the amount of cytokine produced; whereby substitution of G with A at position 1082 results in the production of less IL-10 in vitro (Turner *et al*, 1997). A study examining this polymorphism of IL-10, showed that individuals who retained G at position 1082 were protected against both seroconversion and symptomatic disease (Helminen *et al*, 1999), although it has not been possible to replicate these observations (Dr.

K.McAulay, personal communication). More recently susceptibility to IM and severity of subsequent illness has been linked to HLA class I polymorphisms (McAulay *et al*, 2007). Primary infection in children is not usually accompanied by IM (Fleisher *et al*, 1979). One possible explanation for the age related difference in outcome of primary infection was the suggestion that perhaps young children were infected with a lower dose than young adults. This has been partially countered with the finding of similar viral loads in adults experiencing symptomatic and asymptomatic primary infection (Silins *et al*, 2001). It has been argued that the immune system of children responds differently to that of the young adult. Possible explanations include the lack of clonal expansions of the $CD8^+$ cells of young children, differences in lymphocyte subsets which do not attain adult values until the second decade, and the greater protection offered by the tonsils of younger children which regress after around the age of 14 (Bofill *et al*, 1992; Perry & Whyte, 1998; Selin *et al*, 1998). Racial or behavioural factors might also be important in disease acquisition; IM was not observed in studies conducted in Hong Kong and the Philippines, although seroconversions did occur in young adults (Dan & Chang, 1990; Evans *et al*, 1967). Others have pointed to the role of psychological factors in symptomatic seroconverters; military recruits with high motivation scores combined with poorer academic achievement were more likely to experience IM rather than silent seroconversion (Kasl *et al*, 1979).

The relative contribution of the two types of EBV to IM is not known; Gratama and Ernberg (1995) reported that 7/7 IM cases were cased by type 1 strains. Type 2 is known to have a lesser ability to transform B-cells in culture in vitro (Rickinson *et al*, 1987). The current investigation will investigate the proportion of IM caused by type 2 strains.

Pitfalls in the diagnosis of IM

The typical findings of positive anti-VCA IgG and IgM, positive heterophile antibody and absent anti-EBNA are not found in every case of EBV-induced IM. Anti-VCA IgG and IgM are usually present at the onset of symptoms but one or both may be delayed (Balfour *et al*, 2005; Blake *et al*, 1976; Fleisher *et al*, 1983). Rarely anti-VCA IgM is not detected, which may reflect the low titre of this antibody produced and the screening dilution used (Evans *et al*, 1975). Antibodies to EBNA are usually absent in the acute phase, developing in convalescence, but may occasionally be detected during the first week of illness (Fleisher *et al*, 1983; Henle *et al*, 1974).

Amongst patients with IM, confirmed with specific anti-VCA IgM, only 85-96% will have a positive heterophile antibody test using horse erythrocytes (Blake *et al*, 1976; Evans *et al*, 1975; Fleisher *et al*, 1983). As with anti-VCA antibodies, the onset of detectable heterophile antibodies may be delayed (Blake *et al*, 1976; Fleisher *et al*, 1983). Children aged less than

four are much less likely to have detectable heterophile antibody (Sumaya & Ench, 1985). A more recent review has emphasised that many EBV-induced IM cases will have a normal total lymphocyte count and only 21% had an atypical lymphocytosis comprising 10% or more of the total lymphocyte count (Ventura & Hudnall, 2004).

Laboratory investigations, rather than giving clarity, may add to diagnostic difficulty; false positive heterophile antibody tests have been noted in other infections, notably HIV, connective tissue diseases, lymphoma and in the absence of any illness (Hendry & Longmore, 1982; Horwitz *et al*, 1979; Ridker *et al*, 1990; Seitanidis, 1969; Vidrih *et al*, 2001; Wynne Jones *et al*, 1994). Delayed or atypical serological responses to primary infection may occur in immunosuppressed patients (Gray *et al*, 1992; Ho *et al*, 1985). False positive anti-VCA IgM results have been noted in the presence of rheumatoid factor (Henle *et al*, 1979). This difficulty is further compounded by the fact that rheumatoid factor (IgM to IgG) may be produced in the acute phase of IM (Carter, 1966b). Lastly patients with EBV-induced IM may show a positive result in CMV IgM tests, although patients with CMV apparently do not show positive EBV tests (Horwitz *et al*, 1977).

2.1.2 X-linked Lymphoproliferative Syndrome

X-linked lymphoproliferative syndrome (XLP or XLPS) is a rare familial fatal form of IM which has been recognised for over 30 years (Bar *et al*, 1974; Purtilo *et al*, 1975). The typical clinical presentation is of a young male who is well prior to primary EBV infection but when infected rapidly succumbs to fulminant IM. Death may result from hepatic necrosis, secondary to massive cytotoxic lymphocyte infiltration and cytokine release, aplastic anaemia or pancytopaenia, or, in some cases superimposed bacterial or fungal infection (Mroczek *et al*, 1987; Seemayer *et al*, 1995). Haemophagocytosis is a frequent histological finding (Mroczek *et al*, 1987; Seemayer *et al*, 1995). Other phenotypes including dysgammaglobulinaemia, lymphoproliferative disorders or autoimmune phenomena, including vasculitis, colitis or aplastic anaemia, may arise before or after EBV infection (Nichols *et al*, 2005).

The defective gene is located on the long arm of the X-chromosome and encodes a small cytoplasmic protein known as SH2D1A (Src homology 2 domain-containing protein) or SAP (signalling lymphocytic activation molecule [SLAM] associated protein) (Coffey *et al*, 1998; Nichols *et al*, 1998; Sayos *et al*, 1998). SAP is an adaptor molecule expressed in T-cells and NK cells which binds to the cytoplasmic end of several cell surface proteins including SLAM, 2B4, CD84, Ly9, and NK-T-B-antigen. Thus SAP receives signals from the cell surface and generates cytoplasmic signals, which are thought to regulate aspects of lymphocyte function, including cytokine production, proliferation, cytotoxicity and antibody

formation. If SAP is defective or absent it is thought that the SLAM family of receptors will be unable to signal normally (Nichols *et al*, 2005). To date over 30 mutations of the SAP gene have been identified; currently there does not appear to be a correlation between mutation and phenotype although the complex signalling interactions between SAP and its receptors are not fully understood (Latour & Veillette, 2004; Nichols *et al*, 2005).
XLP is usually fatal, however, a few patients have undergone bone marrow transplantation (Gross *et al*, 1996) and more recently the anti-CD20 monoclonal antibody, rituximab, has been used at the onset of EBV infection in two cases (Milone *et al*, 2005).

2.1.3 Chronic EBV Infection

Chronic active EBV (CAEBV) infection is a rare condition, distinct from chronic fatigue, that is typified by severe, chronic, or recurrent IM-like symptoms after a well documented primary EBV infection, in a previously healthy person. An abnormal antibody pattern is present with greatly increased titres of anti–VCA and early antigen, often accompanied by persisting IgM and IgA to VCA, together with the failure to mount the convalescent phase anti-EBNA antibody. In addition, there is a marked increase of EBV load in the tissues and peripheral blood, and evidence of major organ involvement (e.g., interstitial pneumonia, bone marrow hypoplasia, splenomegaly, extensive lymphadenopathy, hepatitis, meningoencephalitis and uveitis) (Kimura *et al*, 2001; Okano, 2002; Straus, 1988). CAEBV has a high morbidity and mortality from severe fever, hepatic failure, lymphoma, sepsis, cardiac failure or haemophagocytic syndrome. Okano (2002) has proposed diagnostic criteria, which include clinical features, virological and haematological parameters, and, pre-fixing the acronym with "severe", to exclude patients with minor symptoms. The exact pathogenesis is unclear; a perforin defect has been detected in one Caucasian patient, but was not found in an earlier Japanese series, however, clonal expansion of EBV-infected T or NK cells takes place in preference to the infection of B-cells that occurs in normal hosts (Katano *et al*, 2004; Kimura *et al*, 2001; Ma *et al*, 2001). Severe cases are more common in Japan, China and Korea, suggesting a genetic predisposition to CAEBV. The disease is difficult to treat; bone marrow transplantation or adoptive immunotherapy may be helpful (Kuzushima *et al*, 1996; Okamura *et al*, 2000; Savoldo *et al*, 2002).

2.1.4 Burkitt's Lymphoma

This rapidly growing childhood tumour was first recognised as a distinct entity by Denis Burkitt in rural wet lowland areas of equatorial Africa, where malaria was present all year round (Burkitt, 1983). The tumours typically occur in the maxilla or mandible but are usually multifocal, with the retroperitoneal lymph nodes, liver, gut, ovary, kidney and spine

being common sites. The spleen and peripheral lymph nodes are not usually involved. BL with similar clinical and epidemiological features was later recognized in Papua New Guinea and some regions of South America. Subsequently it became clear that tumours with shared histological features occurred at a lower incidence throughout the world without malaria acting as a co-factor (Parkin *et al*, 1985). These latter cases are referred to as sporadic, whilst the malaria-associated associated cases are known as endemic BL. Virtually all (97%) cases occurring where malaria is holoendemic are EBV-associated, and in these areas BL is the commonest childhood malignancy (de-Thé, 1985). Increased availability of chloroquine for treatment of malaria, and programmes of chloroquine prophylaxis in children, appeared to reduce the incidence of BL in endemic areas until chloroquine resistance developed (Geser *et al*, 1989).

The exact role of EBV in the pathogenesis remains unclear despite the strong EBV association in the endemic regions. The tumours characteristically contain clonal EBV episomes and a translocation of the long arm of chromosome 8 in the region of the *myc* proto-oncogene to one of the immunoglobulin gene regions either chromosome 14 (heavy chain) in 80% of cases or, 2 or 22 (light chain) in the remainder (Gulley *et al*, 1992; Magrath, 1990; Zech *et al*, 1976). This results in expression of the translocated c-*myc* which has multiple effects on the cell including growth promotion, stimulation of cell cycle progression and immortalisation. In addition mutations in the tumour suppressor genes p53 and retinoblastoma-like 2 are frequently detected (Küppers, 2003; Young & Rickinson, 2004). During acute malaria increased numbers of EBV-infected B-cells are found in the circulation accompanied by a reduction in EBV-specific cytotoxic T-cell responses (Lam *et al*, 1991; Whittle *et al*, 1984). It is thought that the increased immunological stimulation of repeated attacks of malaria that occur in the regions of intense malaria transmission render errors of the somatic hypermutation process (including translocation of c-*myc*) in the germinal centre centroblasts more likely to occur (Young & Rickinson, 2004) (see 1.9.1 B-Cell Biology).

Sporadic BL is associated with EBV in only about 20% of cases, although in HIV-associated BL the frequency is 35% (Hamilton-Dutoit *et al*, 1993; International Agency for Research on Cancer, 1997). Unlike other forms of Non-Hodgkin lymphoma (NHL) the incidence of BL does not rise with immunosuppression, except that due to HIV; a condition which, like malaria, is associated with chronic polyclonal hypergammaglobulinaemia.

2.1.5 B-Cell Lymphoproliferative Disease

EBV-associated B-cell lymphoproliferative disease (BLPD) occurs mainly in the context of immunosuppression, particularly organ transplantation, where it is known as post transplant

lymphoproliferative disease (PTLD). PTLD is thought to arise in at least 1.4% of transplant recipients, and presents in varied ways including an IM-like illness, and, nodal or extranodal tumours, the latter often involving the gut, brain or transplanted organ (Nalesnik, 1998). Risk factors for PLTD include intensive immunosuppressive drug regimes, often in the context of treating episodes of transplant rejection, primary EBV infection during therapy, and the type of transplant; the highest risks being seen with heart, heart/lung and intestinal grafts (Cockfield, 2001; Ho *et al*, 1985; Nalesnik *et al*, 1997). A spectrum of lesions is seen ranging from polyclonal polymorphic lymphoproliferation, which expresses all EBV latent genes, to biclonal or monoclonal lesions which may present a more restricted pattern of EBV gene expression (Hopwood *et al*, 2002; Rea *et al*, 1994).

Analysis of the heavy chain immunoglobulin genes in PTLD tissue has shown that the disease can arise in variable stages of B-cell development from naïve cells to post germinal centre cells that have immunoglobulin genotypes normally incompatible with B-cell survival, suggesting that EBV facilitates the survival of cells that ordinarily would have been eliminated (Timms *et al*, 2003). Thorley-Lawson has proposed PTLD may arise when cell types other than naïve B-cells, so called bystander cells, become infected with EBV. These cells are unable to differentiate into memory cells, in order to exit the cell cycle, and in the context of a severely impaired cytotoxic response are not eliminated by the immune response (Thorley-Lawson & Gross, 2004). In addition to the background of intense T-cell immunosuppression, the cytokine milieu has also been proposed as important in the pathogenesis of PTLD with elevated levels of IL-4, 6 and 10 thought to contribute, together with reduced secretion of IFN-γ (Burns & Crawford, 2004).

Treatment is difficult; lesions may regress with reduction in the level of immunosuppression, although this threatens the survival of the grafted organ (Starzl *et al*, 1984). As PTLD usually arises in the context of severely impaired cell mediated immunity, infusions of cytotoxic T-cells directed against the EBV latent antigens, derived from either the bone marrow transplant donor, or, a partially HLA-matched blood-donor, have been used in prophylaxis and treatment (Haque *et al*, 2002; Rooney *et al*, 1998). Alternative approaches include use of the humanised mouse monoclonal antibody, rituximab, which targets CD20, depleting both normal and malignant pre-B and mature B-cells; surgical excision; chemotherapy; antibodies to cytokines e.g. anti-IL6; interferon; and radiotherapy (Burns & Crawford, 2004; Cook *et al*, 1999; Faye *et al*, 1998; Haddad *et al*, 2001; Johnson & Glennie, 2001; Kuehnle *et al*, 2000).

2.1.6 Hodgkin's Disease

An involvement of EBV in the pathogenesis of Hodgkin's Disease (HD) had long been suspected because the increased risk following IM, the finding of the typical Reed Sternberg cells of HD in lymph nodes and tonsil excised during IM and raised levels of EBV antibodies prior to diagnosis (Gutensohn & Cole, 1980; Lukes *et al*, 1969; Mueller *et al*, 1989; Mueller, 1996). The epidemiology and EBV-association of HD is complex varying with histological type, socio-economic conditions, geographical location, age and immune status. For many years a paradox existed that the peak of HD occurring in young adults, which was predominantly of the nodular sclerosing subtype, was not EBV-related, however, in 2003 Hjalgrim *et al* were able to demonstrate that the increased risk of HD after IM related specifically to EBV-positive cases of HD, and quantified the risk of developing HD following IM at around one case per 1000 cases of IM (Hjalgrim *et al*, 2003). That IM is not universally followed by HD suggests that other factors, such as the genetic makeup of the host, play an important role pathogenesis (Diepstra *et al*, 2005).

The histology of HD is unusual in that the number of malignant cells, known as Reed – Sternberg and Hodgkin cells (HRS cells) constitute less than 1% of the tumour mass but they are surrounded by a marked, seemingly ineffectual, inflammatory response (Marshall *et al*, 2004; Poppema & van den Berg, 2000). Overall about 40% of cases are EBV-associated, but this figure is much higher for HD occurring at the extremes of age, or in economically-disadvantaged communities, or in Asian or Hispanic, but not black, racial groups (Glaser *et al*, 1997). In the EBV-associated cases clonal EBV episomes are found in the HRS cells (Anagnostopoulos *et al*, 1989; Weiss *et al*, 1989). The rate of EBV positivity varies with histological subtype being most strongly associated with mixed cellularity (70%), followed by lymphocyte depleted (55%), nodular sclerosing (23%), and least associated with lymphocyte predominant (16%), which has now been subdivided into classical and nodular forms (Glaser *et al*, 1997; Yung & Linch, 2003). EBV latent gene expression is confined to EBNA1, EBERs, LMP1 and LMP2 (Pallesen *et al*, 1991).

Irrespective of EBV-status HRS cells are derived from the clonal proliferation of a single germinal centre or post germinal centre B-cell, as evidenced by rearrangement of their immunoglobulin (Ig) genes (Marafioti *et al*, 2000). The HRS cells do not show lethal, non-functional rearrangements of their Ig genes but have lost Ig transcription due to defects in the regulatory genes (Marafioti *et al*, 2000). In EBV positive tumours it is likely that by accident of the presence of EBV these abnormal B-cells, ordinarily destined for apoptosis, receive survival signals.

In the context of HIV, HD is not an AIDS-defining diagnosis although the age adjusted

relative risk for HIV-infected individuals is 11.5 times greater than that of the general population (Frisch *et al*, 2001). HD affects 13% of HIV-positive patients and is EBV-associated in 80-90% of cases (Glaser *et al*, 2003; Powles & Bower, 2000; Uccini *et al*, 1989). Within the tumours detection of EBV type 2 is more common than in HIV-negative patients. HD in HIV generally presents at a more advanced stage and there is a significant excess of the less favourable histological types, mixed cellularity and lymphocyte depleted (Frisch *et al*, 2001; Powles & Bower, 2000). To date HAART has not reduced the incidence of HD, in fact a recent study suggests that the incidence may be increasing, as a survivor effect. Effective anti-retroviral therapy has, however, improved survival in HIV-infected persons that contract HD (Clifford *et al*, 2005; Hoffman *et al*, 2004; International Collaboration on HIV and Cancer, 2000).

2.1.7 AIDS Related Non-Hodgkin Lymphoma

Patients with HIV infection have a risk of NHL at least sixty times greater than that of the general population (Beral *et al*, 1991). The magnitude of the excess risk varies with the different histological types of tumour; being greatest for NHL that arise in the central nervous system (CNS), which is 3600 times more common in HIV-infected persons (Coté *et al*, 1996; Coté *et al*, 1997). Prior to the availability of highly active anti-retroviral therapy (HAART) approximately 16% of HIV-infected patients in the UK had developed a primary CNS lymphoma by the time of death (Peters *et al*, 1991). This figure is thought to be much lower in countries without good supportive medical care, where survival following the onset of AIDS is less, although a post-mortem study in Côte d'Ivoire showed that 4% of adults with AIDS had NHL present at the time of death and 1.7% had primary CNS lymphoma (Lucas *et al*, 1994). In countries where HAART is available, the incidence of some types of HIV-associated NHL appears to be falling (Clayton *et al*, 2006; International Collaboration on HIV and Cancer, 2000; Kirk *et al*, 2001; Polesel *et al*, 2008), although the risk does not return to the population baseline, and concerns remain that the overall prevalence of HIV-associated cancers will increase as survival with HIV improves. In addition, even in countries where HAART is readily available many patients continue to present late with advanced immunosuppression.

NHL arising in the context of HIV are almost always proliferations of B-cells and fall into three main types – Burkitt-like, small, non-cleaved NHL, which tend to occur earlier in the course of HIV infection when the CD4 count is still relatively preserved (median CD4 120-168 (Kirk *et al*, 2001)); large cell or diffuse NHL which tend to occur later (median CD4 count 70), often after other AIDS defining conditions have arisen; and, the rare primary effusion lymphoma which is discussed separately below (2.1.8). The Burkitt-like

lymphomas are associated with EBV in 34% of cases, whereas 77% of the large cell/immunoblastic lymphomas contain EBV (Hamilton-Dutoit *et al*, 1993). Large cell lymphomas affecting the CNS tend to occur at the lowest CD4 counts (median at presentation 15) and are almost-always EBV-related (Hamilton-Dutoit *et al*, 1993; Kirk *et al*, 2001; MacMahon *et al*, 1991). In these cases EBV DNA is detectable in CSF and forms a useful diagnostic test in the evaluation of HIV-positive patients with space occupying lesions on cerebral imaging (Cinque *et al*, 1993). The detection of EBV DNA in the CSF may precede the development of a macroscopic lesion (Al-Shahi *et al*, 2000), however, more recent reports have emphasised the low positive predictive value of this test in HIV-infected patients with neurological disease, including focal brain lesions (Corcoran *et al*, 2008; Ivers *et al*, 2004).

Hodgkin's Disease occurring in the context of HIV is discussed above (2.1.6).

2.1.8 Primary Effusion Lymphoma

Primary effusion lymphoma predominantly arises in the setting of advanced HIV infection but occasionally arise in HIV-negative persons (Boshoff & Weiss, 2001). Malignant cells are found in serosal cavities but without any tumour mass or lymphadenopathy, possibly because they lack adhesion molecules and lymph node homing markers (Boshoff & Weiss, 2002). Malignant cells show dual infection with HHV8 and EBV in about 70% of cases; the remainder are infected with HHV8 alone (Boshoff & Weiss, 2002). The EBV pattern of gene expression resembles that seen in endemic BL, although c-*myc* is not translocated.

2.1.9 T-Cell Lymphomas

EBV DNA has been detected in different types of T-cell lymphoma. Angioimmunoblastic lymphadenopathy and sinonasal angiocentric T-cell NHL, previously known as lethal midline granuloma, show the most consistent EBV association; clonal episomes are detected in almost every malignant cell with a type 2 pattern of latency (Epstein & Crawford, 2005; Jones *et al*, 1988). Approximately 50% of peripheral T-cell lymphomas are EBV associated.

2.2 Diseases of Epithelial Cell Origin

2.2.1 Oral Hairy Leukoplakia

This corrugated white lesion on the lateral borders of the tongue occurs in states of significant immunosuppression including advanced HIV infection (Greenspan *et al*, 1987; Greenspan *et al*, 1985), organ transplantation or occasionally systemic glucocorticoid therapy for other conditions (Flückiger *et al*, 1994; Greenspan *et al*, 1989; Itin *et al*, 1988). As it is a productive (lytic) EBV infection (Thomas *et al*, 1991) it may be controlled with

oral aciclovir therapy (Resnick *et al*, 1988). No EBER expression is seen (Gilligan *et al*, 1990).

2.2.2 Nasopharyngeal Carcinoma

This epithelial cell tumour accounts for 0.7% of the world cancer burden. The distribution shows marked geographical restriction with 44% of all cases occurring in China and 23% in SE Asia (Parkin, 1998). Other populations with raised incidence include North African and Eskimo populations (Wei & Sham, 2005). It is predominantly a tumour of males with a male-female ratio of 3:1 (Le Roux & Joab, 1998). Of the three histological types recognised undifferentiated (WHO type 3) and non-keratinising (WHO type 2) are the most strongly EBV-associated, whereas, squamous cell carcinoma (WHO type 1) is less consistently associated with EBV (Pathmanathan *et al*, 1995a; Raab-Traub, 2002). The prevalence of the histological types also varies geographically. In North America around 25% of patients have type 1, 12% type 2 and 63% have type 3, whereas in South China, the distribution is 2%, 3% and 95% respectively (Wei & Sham, 2005). Migration studies implicate both environmental and genetic contributions to pathogenesis (Buell, 1974) with the ingestion of nitrosamine-containing preserved foods, particularly salted fish, proposed as an environmental risk factor (Buell, 1974; Yu *et al*, 1986; Yu & Yuan, 2002). An increased risk of NPC has been associated with certain HLA types and for individuals that were homozygous for a particular cytochrome P450 polymorphism (Le Roux & Joab, 1998). Elevated titres of EBV antibodies are common in patients with nasopharyngeal carcinoma, with IgA to early antigen and viral capsid antigen described as being characteristic (Henle & Henle, 1976; Old *et al*, 1966; Wara *et al*, 1975). The rise in EBV antibodies predates tumour onset, however, the cost and risk benefit of using antibody-based screening on a whole population basis awaits further study (Chien *et al*, 2001). Quantitative serum EBV DNA may have a role in early diagnosis and disease monitoring, although only 67% of patients with local disease recurrence showed a rise in EBV DNA (Chan *et al*, 2004; Wei & Sham, 2005). Clonal EBV is consistently present in malignant cells and is also found in high grade pre-malignant lesions, suggesting that EBV is involved from an early stage in carcinogenesis, although not necessarily representing the first step in the pathogenesis (Pathmanathan *et al*, 1995b; Raab-Traub & Flynn, 1986).

2.2.3 Gastric Carcinoma

Gastric carcinoma is the fourth most common cancer worldwide and ranks second as a cause of death from cancer (Parkin *et al*, 2005). EBV is associated with around 10% of all gastric carcinomas, although the frequency of this association varies worldwide (Takada, 2000).

Tumours of the lymphoepithelioma-type with a marked lymphocytic infiltrate show the closest association with EBV (90%) and in addition around 10% gastric adenocarcinomas also appear to be EBV-associated (Osato & Imai, 1996; Shibata *et al*, 1991; Shibata & Weiss, 1992), although the histological distinction between these types is not always straightforward (Young & Murray, 2003). In EBV-associated cases all malignant cells contain clonal EBV. The EBV genes expressed are EBNA1, EBERs, LMP2A, and BARF0 (Iwakiri & Takada, 2005; Niedobitek *et al*, 2001; Takada, 2000; Young & Murray, 2003).

2.2.4 Other Lymphoepitheliomas

The term lymphoepithelioma refers to malignant epithelial cell tumours with marked lymphocytic infiltration similar to WHO type 3 NPC. EBV has been associated with lymphoepitheliomas of the thymus (Dimery *et al*, 1988; Leywraz *et al*, 1985), tonsil, salivary gland (Raab-Traub *et al*, 1991), lung, and some gastric carcinomas.

2.2.5 Breast Carcinoma

Breast carcinoma is the commonest malignancy in women and its incidence is increasing (Parkin, 1998). It has been suggested that EBV may be involved in the pathogenesis of some breast cancers (Bonnet *et al*, 1999; Labrecque *et al*, 1995) although this has been refuted by others (Chu *et al*, 2001; Glaser *et al*, 1998; Herrmann & Niedobitek, 2002). In order to explain the conflicting data it has been suggested that EBER negative breast carcinoma may occur, analogous to some cases of NPC, (Magrath & Bhatia, 1999) or that EBV associated breast carcinoma is restricted to certain epidemiological groups (Glaser *et al*, 2004).

2.3 Other Disease Associations

2.3.1 Leiomyosarcoma

EBV has been associated with clonal or biclonal tumours of smooth muscle in paediatric liver transplant recipients (Lee *et al*, 1995) and in young people with advanced HIV infection (McClain *et al*, 1995). Later case reports have described the tumour in cardiac and renal transplant recipients (Davidoff *et al*, 1996; Le Bail *et al*, 1996). Many of the tumours are multifocal, and develop in unusual sites including gut, lung and liver. The pathogenesis is unclear; McClain *et al* (1995) found expression of CD21 on the involved muscle cells.

2.3.2 Multiple Sclerosis

Epstein-Barr virus is one of several agents proposed as having an aetiological role in the pathogenesis of multiple sclerosis (MS). A case-control study in the UK using a population-based database found preceding IM was associated with an odds ratio of 5.5 for the subsequent development of MS (Marrie *et al*, 2000). Distinct subtypes of MS have been

recognized (Lucchinetti *et al*, 1996) raising the possibility that the association is restricted to a subset of cases. A Canadian study identified 27 monozygotic (MZ) twin pairs and 43 dizygotic (DZ) twin pairs with multiple sclerosis. Concordance was 31% for the MZ twins and 5% for the DZ twins from which the authors concluded that although genetic factors were important in determining the susceptibility to MS the findings "unambiguously demonstrates [sic] the powerful effect of non-heritable factors" as the concordance in MZ twins only reached 30% despite prolonged follow-up (Ebers *et al*, 1986; Sadovnick *et al*, 1993). Two years later the same authors published an investigation of MS in adopted children and their relatives, finding that the prevalence of MS among non-biological first-degree relatives was similar to that in the general population, which led them to dismiss any contribution in pathogenesis from "a transmissible agent or any familial microenvironmental factor" (Ebers *et al*, 1995). More recently a Danish population-based study examined the subsequent occurrence of MS amongst over 25 000 patients diagnosed with IM. This study involved more than 550 000 person-years of follow-up and found a more than 2 fold increase in the risk of MS after IM (Nielsen *et al*, 2007).

2.4 Vaccination

Whilst EBV infects most of the human population and co-exists without causing disease in the majority, it is an oncogenic virus that is an established factor in the aetiology of many human cancers. In addition, primary EBV infection is a life threatening condition for those with genetic defects, such as XLP, and for those with profound immunodeficiency following organ transplantation. Furthermore, IM remains an important cause of morbidity in older children and young adults, occasionally causing very severe disease, especially in older age groups. Economic development in Asia, China and South America may result in a rising incidence of IM due to delays in the age of primary infection.

The traditional approach to immunisation, using a live-attenuated virus, has been avoided by researchers in view of the known oncogenic potential of EBV. Two main types of recombinant vaccine are being developed for prophylactic use prior to the occurrence of any disease. These include the use of the subunit glycoprotein gp350, which is known to induce neutralising antibody, and, a peptide (epitope) vaccine, so called "polytope" when multiple peptides are used, designed to induce cytotoxic cellular immunity (Moss *et al*, 1998). Due to HLA diversity it is estimated that 25 $CD8^+$ T-cell epitopes would be needed to provide coverage for >90% of the population in Western countries (Elliott *et al*, 2008).

The use of gp350 as a subunit vaccine has long been considered, and was previously studied in the cottontop tamarin primate model. Following intraperitoneal and intramuscular

injection of EBV these small Colombian monkeys develop large malignant lymphomas which could be prevented by prior injections with gp350 presented in artificial liposomes. Only the animals with very high levels of virus neutralising activity in their serum were protected (Epstein *et al*, 1985).

A small study in China used vaccinia virus that expressed gp350 to immunise 9 children prior to primary EBV infection. After 16 months follow-up all ten controls and three of the vaccine recipients had been infected with EBV (Gu *et al*, 1995). This illustrates the potential for an immunogenic vaccine to delay primary infection, however, concerns remain regarding the widespread use of vaccinia vaccines in view of the relatively high frequency of adverse events and the potential for person to person spread (Frey *et al*, 2002; Sepkowitz, 2003).

Gp350 was subsequently expressed in Chinese hamster ovary cells, and after a delay of over 25 years trials have been undertaken in Belgium with a gp350 subunit vaccine with adjuvant to investigate whether IM can be prevented (Denis *et al*, 2004; Jackman *et al*, 1999). Fortunately there is little sequence variation in this gene between the two EBV types (Lees *et al*, 1993). One hundred and eighty one seronegative young adults in Belgium were randomised to receive either three doses of gp350 with a lipid and aluminium hydroxide adjuvant, or, three doses of a placebo containing aluminium hydroxide alone. Asymptomatic primary infection was not reduced by vaccination with gp350, however, the number of cases of IM was reduced in the gp350 group (2 cases vs. 8 in the placebo group which was significant by intention to treat analysis) (Sokal *et al*, 2007). Unfortunately Sokal *et al* did not investigate viral load or cytotoxic T-cell responses in either group. In an earlier phase I study of gp350 with the same adjuvant, one subject, who was already EBV seropositive prior to study entry, developed oligoarthritis after the second dose of vaccine which might suggest that vaccination would need to be limited to EBV seronegative subjects to reduce adverse reactions (Moutschen *et al*, 2007). A further theoretical concern is that vaccination may increase the numbers of cases of severe IM in older adults by delaying primary infection in into middle age, when IM is associated with more severe disease (Horwitz *et al*, 1983).

To date trials to prevent IM using a polyepitope vaccine have not been conducted. A very small phase I study using a single EBV epitope mixed with tetanus toxoid and formulated in a water in oil adjuvant "Monatide ISA 720" has been conducted in 10 HLA B*0801 positive subjects and 4 controls. Half of the epitope recipients and 2/4 of the placebo recipient seroconverted to EBV. One case of IM occurred in each group. The study was too small to estimate vaccine efficacy, however, the authors concluded that the vaccine was safe, produced epitope specific T-cell responses in 8/9 recipients and was generally well tolerated (Elliott *et al*, 2008). The decision to incorporate tetanus toxoid to provide $CD4^+$ T cell help

was questionable as this probably led to increased local adverse reactions to the vaccine and excluded 45% of potential volunteers, due to pre-exiting high levels of tetanus toxoid antibodies.

Of the two approaches the gp350 would seem the most promising for further trials at the present time, although epitope based approaches may find a role as adjuvant therapies in EBV-associated malignancies (Duraiswamy *et al*, 2003).

3 EBV-negative Glandular Fever

IM caused by EBV is confirmed in 20-25% of patients where it is clinically suspected and laboratory investigations are instigated (Blake *et al*, 1976; Fleisher *et al*, 1983; Wahren *et al*, 1969). The remainder have "IM-like" illnesses which are a heterogeneous group and may include CMV mononucleosis, adenovirus infection, streptococcal infection, influenza, HHV-6 infection, toxoplasmosis, HSV infection, drug reactions, parvovirus, HIV-seroconversion, viral hepatitis, rubella and others (Blake *et al*, 1976; Ho-Yen & Martin, 1981; Rosenberg *et al*, 1999; Steeper *et al*, 1990; Tsaparas *et al*, 2000; Wahren *et al*, 1969). There is overlap in the clinical presentation and haematological features of acute EBV infection and other conditions, although the likelihood of any individual feature being present varies according to the diagnosis. For example CMV mononucleosis is characterised by prolonged fever, lymphocytosis, atypical lymphocytes and abnormal liver function tests. Sore throat and cervical lymphadenopathy are said not to be characteristic of this illness but they do occur in around in a third and a fifth of cases respectively (Horwitz *et al*, 1986; Nankervis & Kumar, 1978; Paublini *et al*, 1977). Paublini *et al* (1977) compared the clinical features in patients with IM, CMV mononucleosis and acute toxoplasmosis; finding that throat involvement, enlarged nodes, fever and splenomegaly were common to all three conditions. Toxoplasmosis was characterised by the presence of lymphadenopathy and the absence of hepatomegaly. Sore throat, fever and splenomegaly occurred only occasionally. Cytomegalovirus infection was characterised by the presence of fever with the other features occurring less often.

Like EBV-induced mononucleosis other acute infections including acute HIV, CMV, toxoplasmosis and hepatitis B may induce severe perturbations of lymphocyte subsets with inversion of the normal CD4: CD8 ratio (Bossi & Bricaire, 2004; Carney *et al*, 1981; Thomas *et al*, 1982; Tindall *et al*, 1988).

4 Fatigue and its Measurement

Fatigue has been described as a negative feeling experienced in relation to an actual or anticipated task (although the performance of a task is not a pre-requisite for the feeling) (Wessely *et al*, 1999). Fatigue is thought to have a number of dimensions including ability to sustain physical and mental functions, a feeling state, underlying biochemical, physiological and psychological mechanisms and its context of cultural factors, social stressors and physical stressors e.g. temperature and noise. Fatigability when defined as the inability to sustain power can be measured electrophysiologically, but this is not necessarily related to the subjective sensation of fatigue. There is no gold standard in fatigue measurement, indeed many fatigue scales have not been tested in chronic fatigue syndrome (Friedberg & Jason, 1998). Fatigue intensity is typically measured by one of three main types of scale: verbal rating (VRS) or Likert-type scale, visual analogue scale (VAS) or numerical rating scale (NRS). A VRS uses list of adjectives to describe fatigue intensity such as mild, moderate etc. A VAS consists of a line, usually 10 cm long, whose ends define the extremes of fatigue "none" to "as bad as it could be". The subject marks the line at the point which represents their fatigue severity and the distance along the line in mm is the recorded measure of their fatigue. The NRS is similar, but easier to administer, and comprises a 0 to 10 or 0 to 100 scale, where 0 is "no fatigue" and 10 or 100 is " as bad as it could be". These types of scales are used to investigate different aspects of fatigue, for example, Lee *et al* used an 18 question/symptom VAS format to investigate fatigue in patients with sleep disorders (Lee *et al*, 1991).

5 Methods

5.1 Chemical Abbreviations

BSA	Bovine serum albumin
Cy-chrome	Phycoerythrin-cyanine5 conjugate
dATP	Deoxyadenosine 5'triphosphate
dCTP	Deoxycytidine 5'triphosphate
dGTP	Deoxyguanosine 5'triphosphate
DMSO	Dimethylsulphoxide
dTTP	Deoxythymidine 5'triphosphate
EDTA	Ethylene diamine tetra-acetic acid
EtBr	Ethidium bromide
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
HBSS	Hanks balanced salt solution
HCl	Hydrochloric acid
KCl	Potassium chloride
KH_2PO_4	Potassium di-hydrogen orthophosphate
$MgCl_2$	Magnesium chloride
NaCl	Sodium chloride
$Na_2H\ PO_4$	Di-sodium hydrogen orthophosphate
PBS	Phosphate buffered saline
PE	Phycoerythrin
RPMI	Rosewell Park Memorial Institute Medium 1640

TE	Tris-EDTA
Tris	Tris (hydroxymethyl) aminomethane
Tween-20	Polyoxethylene (20)-sorbitan monolaurate

5.2 Suppliers

British Drug Houses Ltd.	VWR International Ltd., Hunter Boulevard, Magna Park, Lutterworth, Leicestershire, LE17 4XN.
Becton Dickinson	BD Biosciences, 21 Between Towns Road, Cowley, Oxford, OX4 3LY.
Bioline Ltd	Unit 16, Humber Road, London, NW2 6EW.
Biometra	Rudolf-Wissell-Straβe 30, 37079, Goettingen, Germany.
Bion	ati atlas ltd., The Grange, Church Road, Chichester, PO20 6JW.
DAKO	DakoCytomation, Denmark House, Angel Drove, Ely, Cambridgeshire, CB7 4ET.
Dynex Technologies	Columbia House, Columbia Drive, Worthing, BN13 3HD.
Falcon	Farenheit Laboratory Supplies, 21 Alston Drive, Bradwell Abbey, Milton Keynes, MK13 9HA.
Fischer Scientific UK Ltd	Bishop Meadow Road, Loughborough, Leicestershire, LE11 5RG.
GE Healthcare	Amersham Place, Little Chalfont, HP7 9NA.
Gibco	Invitrogen Ltd., 3 Fountain Drive, Inchinnan Business Park, Paisley, PA4 9RF.
GraphPad	11452 El Camino Real, #215 San Diego, CA 92130 USA.
Invitrogen	3 Fountain Drive, Inchinnan Business Park, Paisley, PA4 9RF.
Machine Shop Equipment Ltd.	MSE (UK) Ltd., (Henderson Biomedical Ltd.), 97 Avenue Road, Beckenham, Kent, BR3 4RX.
MP Biomedicals	Doornveld 10, 1731 Asse Relegem, Belgium.

Leitz	Leitz, Ernst-Leitz Strasse 17-37, Wetzlar, D-35578, Germany.
Microgen	1 Admiralty Way, Camberley, Surrey, GU15 3DT.
Minitab	Quality Plaza, 1829 Pine Hall Road, State College, PA 16801-3008, U.S.A.
MWG Research	MWG Biotech Ltd, Hibernian House, 80a South Mall, Cork, Ireland.
Nunc	Nalge Nunc International, Unit 1a, Thorne Business Park, Hereford, HR2 6JT.
Olympus	Vision House, 19 Colonial Way, Watford, WD24 4JL.
Promega	Delta House, Chilworth Science Park, Abingdon, Oxon, OX14 3NB.
Qiagen	Qiagen House, Fleming Way, Crawley, RH10 9NQ.
Roche	Roche Diagnostics Ltd, Bell Lane, Lewes, BN7 1LG.
Sigma	Sigma-Aldrich Co. Ltd., The Old Brickyard, New Road, Gillingham, SP8 4XT.
Sigma-Genosys Ltd	Sigma-Aldrich House, Homefield Business Park, Homefield Road, Haverhill, CB9 8QP.
Sarstedt Ltd.	68 Boston Road, Leicester, LE4 1AW.
Scientific Laboratory Supplies	Unit 17, Coatbridge Business Centre, 204 Main Street, Coatbridge, Lanarkshire, ML5 3RB.
Sterilin	Sterilin Ltd., Angel Lane, Aberbargoed, Bargoed, Caerphilly, CF81 9FW.
Stratagene	11011 North Torrey Pines Road, La Jolla, CA 92307, US.
Sysmex UK Ltd	Sunrise Parkway, Milton Keynes, MK14 6QF.
Ultra Violet Products Ltd	Unit 1, Trinity Hall Farm Estate, Nuffield Road, Cambridge, CB4 1TG.

5.3 General Plastic Ware

Plastic ware was supplied by Scientific Laboratory Supplies or Sterilin unless otherwise

stated, except for cryovials (Nunc) and eppendorfs (Sarstedt).

5.4 Recruitment to Longitudinal Cohort Study

Students commencing four year courses at Edinburgh University in October 1999 and October 2000 were invited to join a longitudinal study to investigate the epidemiology of EBV. An information leaflet and posters were produced. Students registering at the University Health Centre (UHC) at the start of their university course who agreed to join the study were given further verbal explanation regarding the study aims and requirements, and were allocated a unique study number. Thereafter each student signed a consent form, completed an anonymised family and lifestyle questionnaire and gave a venous blood sample. A few students preferred to donate a smaller capillary blood sample. All students were sent a letter of thanks informing them of their serology result. Seronegative students were advised to contact the UHC if they developed symptoms of IM. Students who developed IM during their course were asked to attend for follow-up to document their illness in more detail; these students additionally joined the case-control study. At the end the autumn term in their final year at university (December 2002 and 2003) all students participating in the longitudinal cohort study (LCS) were invited to re-attend the UHC to complete a further anonymised lifestyle questionnaire and to donate a further venous or capillary blood sample. Students were contacted by personal email and additional publicity was generated by means of posters and articles in the student newspaper. In order to maximise follow-up students were offered a cinema ticket or book token as a thank you gesture for attending the follow-up session.

5.5 Questionnaires

The anonymous questionnaires were formulated jointly with Professor Anthony Swerdlow and Craig Higgins, epidemiologists at The London School of Hygiene, taking account of the approaches used by the successful *National Survey of Sexual Attitudes and Lifestyles* (Wellings *et al*, 1994). Data were gathered on family background and living circumstances, ethnicity, previous illness, life events, sexual relationships, smoking, alcohol and exercise history. The questionnaires are in the appendix (12.1). A further questionnaire was administered if a student developed IM and collected information about living circumstances, life events and illnesses, sexual relationships, travel and immunisations, smoking, alcohol and exercise history at university. The questionnaire at the end of the study was similar to that used if a student developed IM and concentrated on information

relating to the period of time at university. Additionally questions relating to possible undetected glandular fever illness were included.

5.6 Recruitment of IM Cases to the Case Control Study

At recruitment to the LCS students were asked to contact the study if they developed glandular fever. The EBV negative students were also reminded to do this in the letter that they received informing them of their serology result at the start of the study. Additionally a diagnostic serology service for suspected IM was provided to the general practitioners at the UHC, where most of the students in the LCS received their health care. Students with diagnostic serology compatible with acute IM who had already been recruited to the LCS were invited to attend for clinical follow-up. Additional students with acute IM who were not already recruited to the LCS were given a study information sheet by their general practitioner (GP) at the UHC and invited to attend for clinical follow-up. Seronegative students from the LCS matched for gender, age and year of study were selected by random number by Craig Higgins to act as controls for the IM cases.

Acute IM blood samples were drawn at the time of diagnosis. Convalescent samples were taken at follow-up visits after the diagnosis of IM was made. Where possible a concurrent sample was sent for determination of the total lymphocyte count at The Royal Infirmary of Edinburgh haematology laboratory. Liver function tests were measured at the first visit. Students with abnormal liver function test (LFT) results had these followed up until recovered. Clinical data was recorded on the "Clinical assessment" sheets (see Appendix 12.1) at the time of clinical review, and entered into an encrypted and password protected access database by Ha Nguygen. Student identity was by use of the study number only.

We measured physical and mental functions by asking questions about walking distance, perceived ability to concentrate, whether the student left the house when unwell and whether they got washed and dressed during their most severe symptoms. In addition students completed questions about the duration of time spent sleeping, studying, attending university, exercising, and on non-exercise related social activities (see "Physical Function and Fatigue", Appendix 12.1). A numerical rating scale from zero "no fatigue" to ten "as bad as you can imagine" based on the brief fatigue inventory gave a numerical score to the student's perception of their fatigue severity at each visit (Mendoza *et al*, 1999).

Students were reviewed as soon as possible after diagnosis, at two months and again at 6 months after diagnosis. As the parental homes of most students were distant from Edinburgh considerable flexibility was needed because of vacations and students returning to the parental home to recuperate. Students that had not recovered by six months after diagnosis

were contacted by email or telephone to determine the dates of return to social activities, exercise and the duration of their fatigue.

5.7 Sample Processing

5.7.1 Peripheral Blood

Up to 18 ml of peripheral blood was drawn into EDTA containing tubes (Sarstedt) and separated into plasma and peripheral blood mononuclear cells (PBMC) by density gradient centrifugation. The PBMC were used for flow cytometry and a portion was stored at –70 °C as a source of DNA for viral load estimation and strain typing.

The EDTA whole blood was layered over an equal volume of Ficoll (Histopaque_1077, Sigma) as described by Böyum (Böyum, 1968) in 50 ml conical-bottomed tubes (Falcon). These were centrifuged (MSE [Machine Shop Equipment Ltd.] Mistral 3000i) at 900g for 15 minutes at 20°C. After separation by centrifugation 3 ml of plasma was retained for serological testing. The layer of peripheral blood mononuclear cells, located between the plasma and the Ficoll, was removed by aspiration, placed in a second conical-bottomed tube and washed, in Hanks balanced salt solution [without calcium and magnesium] (HBSS) (Gibco), by spinning at 270g for 7 minutes at 4°C. This supernatant was discarded and the cells were re-suspended in fresh HBSS. Viable cell counts were performed by mixing an aliquot of the re-suspended cells with an equal volume of 0.4% trypan blue (MP Biomedicals) and counting cells excluding blue dye using a haemocytometer (Improved Neubauer, BDH Ltd.). The concentration of cells was adjusted to $1x10^6$ per ml of tissue culture medium with a portion used for flow cytometry, and the remainder stored in aliquots of $5 x10^6$ cells which were kept in eppendorfs at –70 °C as a source of DNA for viral load and typing assays.

5.7.2 Throat Wash

Throat wash samples were collected by gargling with 10 mls 0.9% sterile saline for 30 seconds. The specimen was centrifuged at 760 g for 10 minutes at 4 °C and the supernatant discarded. The pellet was re-suspended in 3 mls of sterile PBS in a sterile eppendorf and micro-centrifuged (Micro Centaur, MSE) at 1900g for 5 minutes. The dry pellet was stored at –70 °C until required.

5.8 Serological Testing

5.8.1 Heterophile Antibody

Plasma was tested for the presence of heterophile antibody using a rapid slide agglutination test (Microgen i.m. absorption kit). 25 µl of plasma was added to one drop each of ox cell and guinea pig antigen suspensions supplied and mixed thoroughly on a clean glass tile. One drop of the positive control was also mixed with each of the antigen suspensions above. One drop of horse red blood cell suspension was added to each of the 4 ovals on the tile and mixed well. Agglutination in the guinea pig oval, with none in the ox cell oval, at one minute indicates a positive heterophile antibody test.

5.8.2 Detection of anti-VCA IgG and IgM

Serological testing using plasma (Davidson & Main, 1971) was performed after heat inactivation to 56 °C for 30 minutes. IgG and IgM to EBV VCA were detected by indirect immunofluorescence (Henle & Henle, 1966). Slides for the detection of IgG to VCA were prepared in-house using the EBV positive cell line P3HR-1 (Hinuma *et al*, 1967). 10^5 cells were dispensed onto each well of 12 well slides (BDH), dried and fixed in acetone for 10 minutes. Serial dilutions of plasma in phosphate buffered saline (PBS) were made from 1/10 to 1/2560. 20 µl of diluted plasma was added to each well of a 12 well slide starting with the highest dilution. The slides were incubated in a moist chamber for one hour at 37°C and then washed twice with PBS for ten minutes. 15 µl of FITC-conjugated rabbit-anti human IgG (Dako) [diluted 1/50 with PBS] was applied to each well and incubated for 45 minutes at room temperature, before again washing the slides twice in PBS. A negative control and serial dilutions of a positive control (as per the test sample) were included in every batch. Antibody detection for IgM was performed at only one dilution (1/10) using the same method except that commercial slides were used (Bion) and FITC-conjugated rabbit anti-human IgM (Dako) diluted 1/50 with PBS was used as the second layer antibody. Positive and negative (EBV VCA IgG positive, IgM negative) controls were included on every slide at a dilution of 1/10.

Slides were mounted with 50:50 PBS : glycerol and examined under the UV microscope (Leitz Ortholux II) at x400 for the presence of fluorescing cells. For the detection of IgG anti-VCA the titre at which the fluorescence diminished was recorded. For IgM anti-VCA the samples were scored as positive or negative for IgM in comparison with the control wells. Samples with detectable VCA-IgM were additionally tested for rheumatoid factor.

5.9 DNA Extraction

DNA extraction from PBMC and throat gargle pellets stored at – 70 °C was performed using the Invitrogen Easy DNA kit (Invitrogen) according to the manufacturer's instructions. Frozen cell pellets (approximately 5 x 10^6 cells) were thawed in a water bath and then re-suspended in 200µl of PBS. 350 µl of solution A (lysis solution) was added to the cells and mixed until dispersed. The mixture was incubated for 10 minutes at 65 °C. 150µl of solution B (precipitation solution) was added to form a precipitate of proteins and lipids. 500µl of chloroform (Sigma) was added and agitated until the solution was homogeneous. The samples were then micro-centrifuged at 15500 g for 15 minutes at 4 °C which separated each sample into 3 phases. The uppermost, containing the DNA, was recovered into a fresh eppendorf and the DNA was precipitated with 1 ml of 100% ethanol (BDH). After overnight freezing at –20 °C the DNA was micro-centrifuged as before, and the ethanol was removed. The pellet was mixed with 500 µl of 80% ethanol and micro-centrifuged again for 5 minutes under the same conditions. The ethanol was removed and the pellet was left to air dry for 5 minutes, and then re-suspended in 50 µl of nuclease free water. The DNA concentration was measured using a UV spectrophotometer (GeneQuant, GE Healthcare) and aliquots prepared for storage at –20 °C until used.

5.10 PCR

All PCR work was carried out by Karen McAulay. DNA was diluted with nuclease free water to 100ng ml^{-1} for use in PCR and all PCR was carried out on a T3 Thermocycler (Biometra) or a Robocycler 96 (Stratagene). Promega and GE Healthcare reagents were used unless otherwise stated and all primers were obtained from Sigma Genosys. PCR of the β-globin gene was undertaken to verify the quality of DNA extraction. Positive and negative controls were used in all reactions. PCR products were run on 2% agarose gels containing ethidium bromide and visualised under a UV light source (UVP transilluminator, Ultra Violet Products).

5.10.1 Viral Load

Viral load estimation of PBMC stored at – 70 °C was carried out using a quantitative competitive PCR technique following the method of Stevens *et al* (1999). This method is based on competitive co-amplification of a highly conserved 213 bp region of the EBNA1 open reading frame together with an internal standard with an identical amplification efficiency added in known concentrations (Stevens *et al*, 1999). DNA was amplified in a 50µl reaction containing 50mM KCl, 1.5mM $MgCl_2$, 10mM Tris-HCl (pH 9.0), 0.1% triton

X-100, 25 pmol of each primer (5'-GCC GGT GTG TTC GTA TAT GG-3' and the biotin labelled 5'-CAA AAC CTC AGC AAA TAT ATG AG-3', 200 μM of each deoxynucleoside triphosphate and 2U of *Taq* polymerase (Promega). For each sample tested a series of wells were set up containing decreasing numbers of the internal standard (10^6, 10^5, 10^4, 10^3,10^2, 10 and 1 EBV genome copy). The cycling conditions were 5 minutes at 95 °C, 40 cycles of 95 °C for 1 minute, 55 °C for 1 minute and 72 °C for 1 minute, and finally 5 minutes at 72 °C. The PCR products were quantified by enzyme linked immunoassay. Five μl of each product was added to 45μl of PCR buffer in two separate streptavidin coated microtitre plates (Roche) and incubated for 60 minutes at 37 °C. The plates were washed five times with PCR buffer, the DNA was then denatured with 0.2M sodium hydroxide for 15 minutes at room temperature and the plates were washed again twice. The plates were incubated with 50pmol ml^{-1} of the digoxigenin labelled (Roche) wild type probe (5'-TCT CCC CTT TGG AAT GGC CCC TG-3') or the internal standard probe (5'-CTA TAT GCC TGC TTC CTC CGG CG-3') for 60 minutes at 37 °C and then washed three times. The plate was incubated with anti-digoxigenin alkaline phosphatase and incubated for one hour at 37°C. After washing three times the plate was incubated with nitrophenyl phosphate (Sigma) for one hour at 37°C. Optical density was read at 405 nm using a plate reader (MRXII, Dynex). EBV copy number was calculated by plotting the log optical density of the wild type/internal standard against the log of the number of the internal standard copies. The EBV copy number in the sample is taken as the figure where the x-axis is intercepted (see Figure 1).

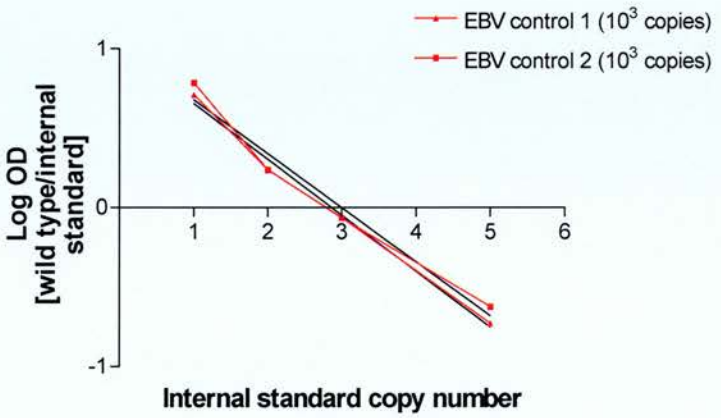


Figure 1 Estimation of EBV copy number

Figure from Karen McAulay:

Log OD [wild type/internal standard] versus internal standard copy number for duplicate EBV control samples containing 10^3 copies of the EBV genome. Linear regression was performed to calculate x-intercept and the copy number calculated as the inverse log of the x-intercept.

5.10.2 Type 1 and 2 Strain Typing

DNA was extracted from stored PBMC as above. The type of EBV in each sample was determined by nested PCR across a type specific region of the EBNA 3C gene (Brooks *et al*, 2000; Sample *et al*, 1990). 500ng –1μg of DNA was amplified in a primary reaction mix containing 1x PCR reaction buffer (50 mM KCl, 10 mM Tris-HCl (pH 8.3) and gelatin 0.01%) together with 1.5 mM $MgCl_2$, 200μM of each deoxynucleotide, 0.1 μM of each primer (5' CAC AGA GCA CCC CTG AAA GG-3' and 5'-GGC TCG TTT TTG ACG TCG GC-3') and 1 unit of *Taq* polymerase (Promega). The cycling conditions were 1 cycle of 94°C for 5 minutes; 40 cycles of 94 °C for 30 seconds, 90 seconds at 45 °C and 120 seconds at 72°C; followed by a final incubation of 72°C for 5 minutes. The primary product (2.5-5 μl) was further amplified in a second reaction mixture with the secondary primers (5'-AGA AGG GGA GCG TGT GTT GT-3' and 5'-GTC TTG ATG TTT CCG ATG TGG CTT A-3') under exactly the same conditions as before. Ten μl of the final products were separated on a 2 % agarose gel (Bioline Ltd) containing ethidium bromide (Sigma) and visualised under UV light (Ultra Violet Products Ltd). The gel was Southern blotted and probed with the digoxigenin labelled 5'-GAA GAT TCA TCG TCA GTG TC-3' (type 1) or 5'-CCG TGA TTT CTA CCG GGA GT-3' (type 2) (Roche).

5.10.3 Strain Typing LMP1 and EBNA3C Repeats

Strain typing was performed by examining the numbers of repeats in two regions each known to carry variable numbers of repeats the 39 bp region of EBNA3C and the 33 bp repeat region of LMP1.

EBNA 3C 39 bp repeat region PCR

The number of repeats in the EBNA3C region varies from 3 to 12 which can be used to demonstrate variation between EBV isolates. This region was amplified using nested PCR as described by Haque *et al* (Haque *et al*, 1996). 500 ng –1 μg of DNA was amplified in a 50 μl primary reaction mix containing 10mM Tris-HCl (pH 8.3), 50 mM KCl, 1 mM $MgCl_2$, 0.01% gelatine together with, 200 μM of each nucleotide, 0.1 μM of each primer (5'-ACA CTT GAG TTC CAT GTC GC-3' and 5'-TGT AAT CAC TGG CAA AGG GC-3') and 1 unit of *Taq* polymerase (Promega). The cycling conditions were denaturation at 94°C for 5 minutes, 30 cycles of denaturation at 94 °C for 30 seconds, annealing at 55 °C for 60 seconds and extension at 72°C for 90 seconds, with a final incubation at 72°C for 10 minutes. 2.5 μl of the product was re-amplified in a 50μl reaction volume using the inner primers 5'-TAT CGC ACG AAG AAC AAC CCC-3' and 5'-AGA TGT GGG AAC TGG GAG ACC-3' for 30 cycles as above except that an annealing temperature of 65 °C was used. Ten μl of the

nested product was run on a 2% agarose gel (Bioline Ltd.), Southern blotted and hybridised with a digoxigenin labelled probe 5'-CAC GGG CTC CAA TCA TCT TC-3'. The number of repeats was compared with known standards previously determined by sequencing.

LMP1 33 bp repeat region PCR

The LMP1 gene contains a variable number of 33 bp repeat sequences which have been used to characterize variation between isolates (Miller *et al*, 1994). The repeat region in the LMP1 gene was amplified following the method of Khanim *et al* using the primers 3'-TTT CCA GCA GAG TCG CTA GG-5' and 5'-GGC GCA CCT GGA GGT GGT CC-3' (Khanim *et al*, 1996). 500 ng –1 μg of DNA was amplified in a 50 μl reaction containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.01% gelatin, 1.5 mM $MgCl_2$, 200 μM of each deoxynucleoside, 1 μM of each primer and 1 unit of *Taq* polymerase (Promega). The conditions were 5 minutes at 94°C, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for 45 s and extension at 72°C for 90 s, with a final incubation of 72°C for 10 minutes. Twenty μl of product was separated on a 2% agarose gel and Southern blotted. A digoxigenin labeled probe 5'-AGG ACC CTG ACA ACA CTG AT-3' was used to identify the LMP1 repeats and the number was compared with known standards (previously determined by sequencing).

Sequencing

In instances where the number of repeats of a given isolate could not be determined on the Southern blot then sequencing was undertaken. PCR products were excised from the gel using QIAquick gel extraction kit (Qiagen). Direct sequencing of the product was performed commercially by MWG Research using the forward primer.

5.11 Flow Cytometry

The following cellular populations were studied: $CD8^+/CD3^+$, $CD4^+/CD3^+$, $CD56^+/CD3^-$, $CD19^+/CD3^-$ and $CD3^+/\gamma\delta^+$.

Immunostaining of mononuclear cells, harvested as above, was performed by aliquoting 2 x 10^5 cells in polystyrene tubes (Becton Dickinson, [BD]) on ice. Ten μl of the required monoclonal antibody (BD) was added to each tube, vortexed, and incubated for 15 minutes in the dark at 4°C. The cells were washed twice with flow cytometry (FCM) buffer by spinning at 200g at 4°C for 5 minutes (MSE Mistral 3000i). Unstained cells and cells mixed with fluorochrome isotype controls (BD) were included for every individual.

Flow cytometry was performed within 4 hours of staining using a BD Facs Caliber (BD). The lymphocyte population was identified on the basis of forward and side scatter

characteristics (see Figure 2). This was then used to identify a population of cells for analysis – a so-called "gate". For each sample 10,000 cells were accumulated into the lymphocyte gate. Data analysis was performed using Cell Quest acquisition and analysis software (BD). The lymphocyte subset composition of each sample was determined by analysing each cell population in turn e.g. $CD4^+$ vs. $CD3^+$ to assess the proportion of cells positive for both markers. Actual lymphocyte numbers for each subset were calculated from the peripheral blood total lymphocyte count obtained at the same time and performed at the Department of Haematology Edinburgh Royal Infirmary using an automated counter (SE-9000, Sysmex).

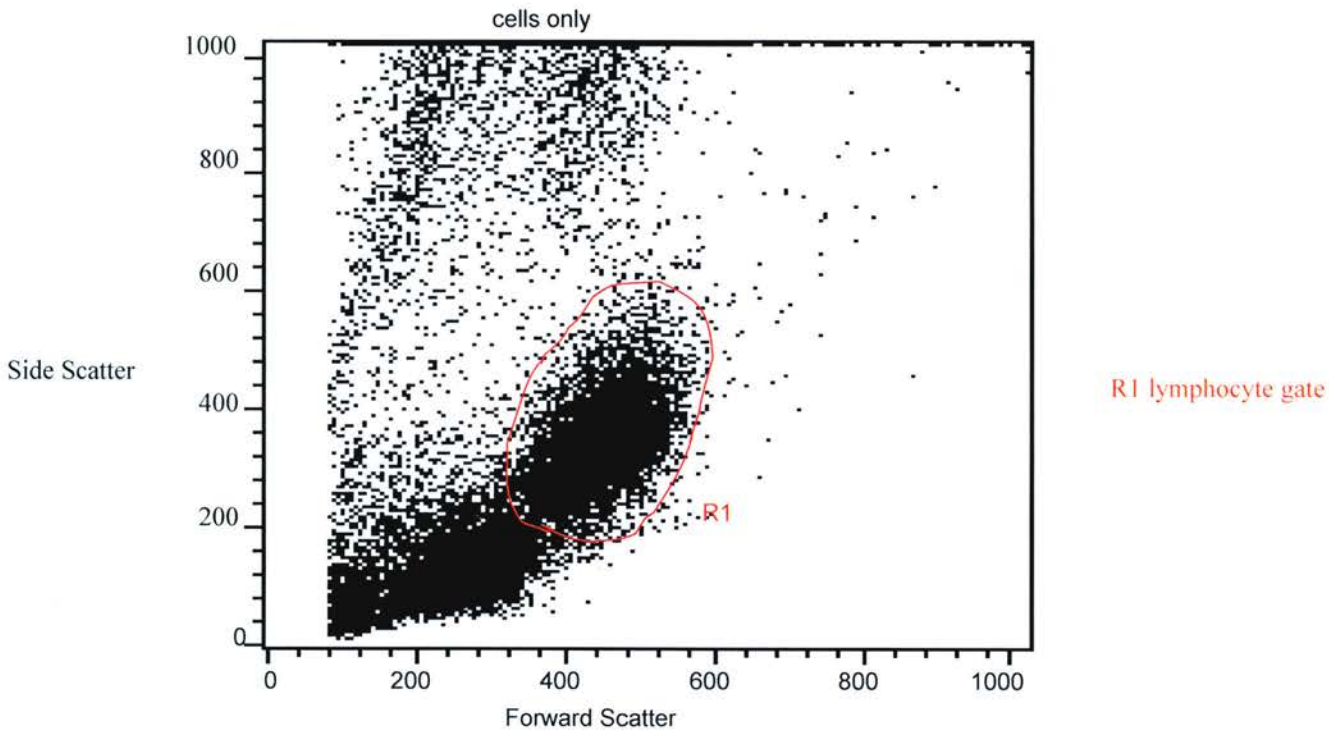
5.12 Ethics

Ethical committee approval was obtained for LCS, the case control study, and the use of remainder samples from the Lothian Region Ethics Committee. In addition the consent was obtained from the Edinburgh University Students Ethics Committee for the LCS and the case control study. All students participating in the studies received a verbal explanation about the aims of the study and a written information sheet, and then signed a consent form.

5.13 Statistical Methods

The Mann Whitney U test was used to compare median values of quantitative variables using Minitab version 13 and GraphPad PRISM software version 4, 2003. Two sample t tests were used to compare the pulse and blood pressure of cases and controls. The Spearman correlation coefficient was used to examine the relationship between two continuous variables using GraphPad PRISM software version 4, 2003. All significance tests are two sided. Two discontinuous variables were compared using the Chi square test if numbers permitted (Minitab version 13) or the Fisher's exact test if any variable had a value of 5 or less (www.langsrud.com/fisher.htm). Multivariate analysis was undertaken by Craig Higgins using a multivariate Poisson model where a number of potential risk factors for EBV seropositivity were included together to determine prevalence ratios (Higgins *et al*, 2007). The prevalence ratios were used to establish which factors were independently associated with a significantly increased risk of EBV infection.

Figure 2
Forward and side scatter characteristics of peripheral blood mononuclear cells showing the lymphocyte gate



6 Longitudinal Cohort Study

6.1 Recruitment

Students were recruited to the four year LCS at the start of two consecutive academic years, 1999 and 2000. Overall 2048 students were recruited to the LCS of which serology was successfully performed on 2006 (98%). This represents just over half of the 4000 students that registered with the UHC at the start of these two academic years. Students following courses lasting less than four years were ineligible. Of the 42 students that were recruited but could not join the study 3 withdrew from their university course, demographic information and consent forms were incomplete in 4, questionnaires were not completed in 2 and the blood sample was not obtained or was insufficient in 33. The age range was 17.0 to 42.7 with a median age of 18.7 years. Sixty three percent (1258) of the recruits were female.

6.1.1 EBV Baseline Serology by Age and Sex

Of the 2006, 75% (1499) had detectable EBV anti-VCA IgG. The median age of the seropositive students was 18.8 years (range 17.0-42.7), which was greater than that of the seronegative students (18.6 years, range 17.4-31.4, $p<0.001$). A significantly higher proportion of female students, 79% (987/1258) were seropositive, as compared with 69% of the males (512/748, $p<0.001$).

6.1.2 Ethnicity

Analysis of ethnicity by white and non-white groups did not shown any significant difference in the proportion of students that were EBV seropositive; 75% (1409/1887) of white students and 76% (86/113) of non-white students had EBV antibodies, ($p=0.73$, Table 6). As there were only 113 non-white students numbers were too small for further analysis.

6.1.3 Residence in a Tropical Country

Students that had resided in a tropical country before university were much more likely to be seropositive (276/341, 81%) than those who had not (1184/1616, 73%, $p=0.003$) (Table 6). For those born in a tropical country the EBV seroprevalence was slightly higher (84%).

6.1.4 Household Crowding

Students were asked to record the number of bedrooms and living rooms in their home prior to university and the number of people sharing these rooms. From this information the average number of rooms per person in the household was calculated for each student. No

association between household crowding and EBV status was detected; the median number of rooms per person for both EBV positive and negative students was 1.8. Students were also asked if they shared a bedroom as a child. There was no difference in the proportions of seronegative or seropositive students that had shared a bedroom prior to university (p=0.110).

6.1.5 Siblings

Seventy five percent (1422/1888) of students with siblings were seropositive as opposed to 66% of those without (75/114, p=0.023). (Table 6)

6.1.6 Breast Feeding

The cohort of students recruited in 2000 were asked how they had been fed as infants. A large proportion (91%) reported that they had been breast fed. No association with EBV status was found (Table 6).

	n	Characteristic present		Characteristic absent		p value
			% EBV seropositive		% EBV seropositive	
Siblings	2002	1888	75	114	66	p=0.023
Crowding > median (1.8 rooms per person)	1934	1003	75	931	75	p=0.674
Breast fed	823	753	75	70	73	p=0.633
Non-white ethnicity	2000	113	76	1887	75	p=0.733
Residence in tropical country	1957	341	81	1616	73	p=0.003
Born in tropical country	2006	127	84	1879	74	p=0.011
History of smoking	1976	443	82	1533	73	p=0.001
Consumes alcohol	1981	1534	76	447	70	p=0.009

n Number of students

Table 6 EBV seroprevalence and baseline characteristics

6.1.7 Previous IM

Eleven percent (213/2006) of students gave a history of prior IM at university entrance. All were EBV seropositive.

6.1.8 Sexual Behaviour

Students that reported a sexual relationship before arrival at university were significantly more likely to be seropositive (944/1133, 83%) than those who had never been sexually active (547/862, 64%, $p<0.001$). There was no significant difference in the proportion of males (55%) and females (58%) that had been sexually active before university ($p=0.143$). Among the 213 students with a previous history of IM 77% had been sexually active, whilst among the seropositive students without a history of IM this figure was 61%, and in the seronegatives 38% had been sexually active. The likelihood of being seropositive was increased with increasing numbers of sexual partners; 78% of students (328/422) with 1 prior sexual partner were seropositive; 85% of students (397/467) with 2 – 4 sexual partners were seropositive and 91% of students (213/235) with 5 or more sexual partners were seropositive, (p trend <0.001) (Table 7). On initial analysis there appeared to be an association between age at first sexual intercourse and the likelihood of EBV seropositivity (Table 7). After stratification of the seroprevalence results by numbers of sexual partners the significant association between age at first sexual intercourse and EBV status was no longer seen (Figure 3).

Condom use

Among the sexually active students those that "always" used a condom were less likely to be seropositive (506/624, 81.1%) than those that "seldom or never" used a condom (404/465, 86.9%, $p=0.011$). (Table 7)

Sexually transmitted infection

Among those who were sexually active 45 (4%) gave a history of a prior sexually transmitted infection. These students were significantly more likely to be seropositive (93%) than other sexually active students ($p=0.018$).

	EBV negative (%)	EBV positive (%)	p value
Sexually active n=1995			
no	315 (37)	547 (63)	
yes	189 (17)	944 (83)	p<0.001
Number of sexual partners n=1986			
0	315 (37)	547 (63)	
1	94 (22)	328 (78)	
2-4	70 (15)	397 (85)	
≥5	22 (9)	213 (91)	p<0.001
Age at first sexual intercourse n=1989			
Never	315 (37)	547 (63)	
<16	24 (12)	178 (88)	
16-17	97 (16)	492 (84)	
≥ 18	65 (19)	271 (81)	p<0.001
Condom use n=1951			
not sexually active	315 (37)	547 (63)	
never/seldom	61 (13)	404 (87)	
always	118 (19)	506 (81)	p=0.011

Table 7 EBV seroprevalence and sexual behaviour

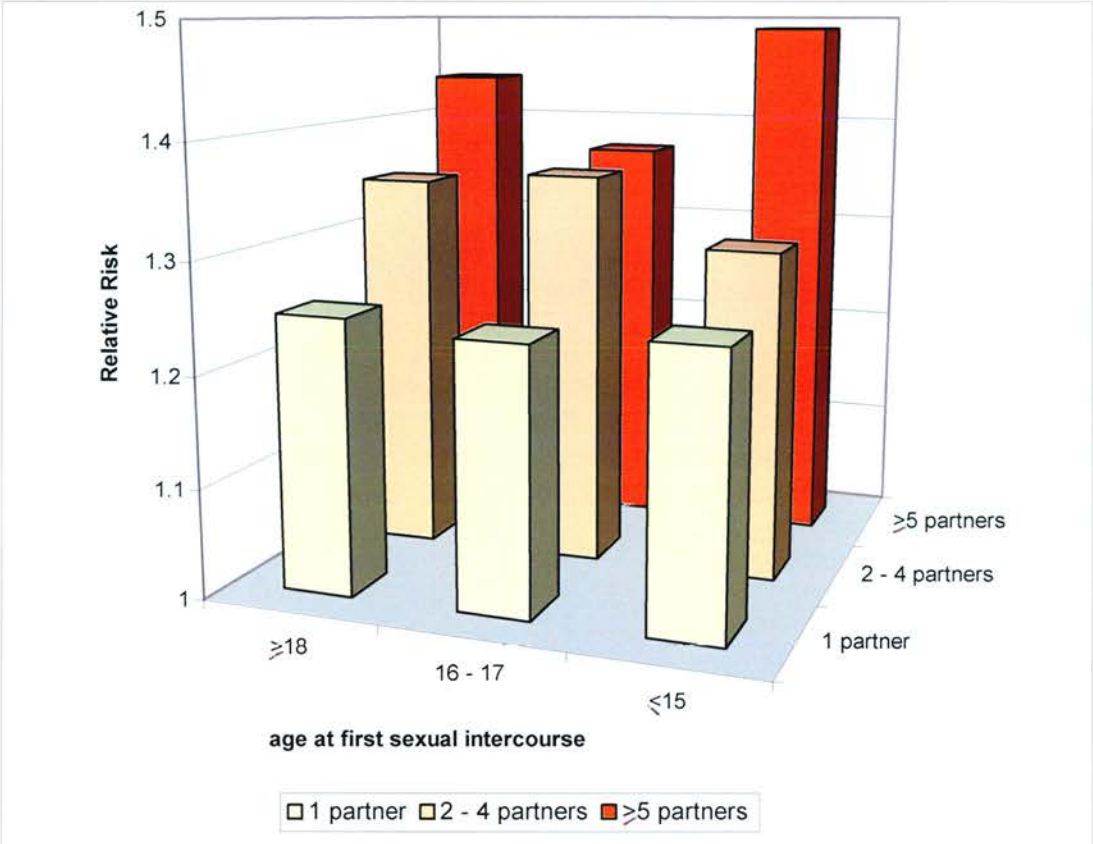


Figure 3 EBV seroprevalence by age at first intercourse and number of sexual partners

6.1.9 Smoking

Students that smoked were more likely to be seropositive 365/443, 83% than those who did not 1111/1533, 73% ($p<0.001$), however after adjusting for sexual behaviour the results were no longer significant.

6.1.10 Alcohol

Students that consumed alcohol were more likely to be seropositive 1168/1534, 76% than non-drinkers 313/447, 70%, ($p=0.009$), however after adjusting for sexual behavior the results were no longer significant.

6.1.11 EBV Strain Types 1 and 2

DNA was successfully amplified using PBMC stored at recruitment in 625 of 1499 (42%) seropositive students. Type 1 EBV was detected in 483 (77%), type 2 was present in 108

(17%) and both types were detected in 34 (5%).

6.2 Serological Follow-up of the Longitudinal Cohort

A total of 842 students donated a follow-up blood sample (443 from the 1999 cohort and 399 from the 2000 cohort) after 3 years at University. Of the initially 510 seronegative students, 241 (47%) completed a further questionnaire about their lifestyle at university and gave a blood sample. Amongst the 241, 38% (91) were male and 62% (150) were female (see Table 8). This is almost identical to the proportions of males and females recruited to the initial cohort overall (37% male, 63% female) but because more males than females were originally seronegative this means that a higher proportion of female seronegative students returned for follow-up, ($p<0.001$). Considering the originally seronegative students there was no significant difference in the sexual behaviour before university between the returning and non-returning students ($p=0.128$).

	Total	Male	Female
Recruited	2006	748 (37%)	1258 (63%)
Seronegative at recruitment	507	236 (47%)	271 (54%)
Seronegative students returned for follow-up	241	91 (38%)	150 (62%)
EBV seroconversion at university	110 (54%)	40	70
% seroconverted by gender		44%	47%

Table 8 EBV Seroconversion at university

6.2.1 EBV Seroconversion and IM at University

Of the 241 students that returned after 3 years 110 (46%) had acquired EBV whilst at university, indicating an annual seroconversion rate of 15%. The likelihood of seroconverting was not significantly different between male and female students (44% vs. 47%, $p=0.682$). The median age of those that seroconverted and those that remained seronegative was 21.6 years. The proportion that had symptomatic acquisition of EBV, diagnosed as IM, was 27/110 (25%) which gives an annual IM rate of 3.8% in seronegatives.

Twelve of the IM cases were male (44%) and 15 (56%) were female, (p=0.447).

6.2.2 Sexual Behaviour at University

Almost all (95%) of the seronegative students that returned for follow-up had some form of sexual experience during three years at University. The median number of sexual partners was 2.5. In 185 of the 241 (77%) this included penetrative intercourse, and these students were significantly more likely to have seroconverted to EBV (51% vs. 29%, p=0.003). Amongst the 185, those that "always" used a condom were less likely to have acquired EBV (42/91, 46%) than those who reported "seldom or never" using a condom (52/92, 55%), although this did not attain significance (p=0.213). Sexually active students who had seroconverted reported a significantly greater number of sexual partners (median 3) than sexually active students who had remained EBV seronegative (median 2, p=0.030).

7 Results Case Control Study

7.1 Recruitment to the Case Control Study

Sixty cases of IM, 28 males and 32 females, were recruited to the case control study via the UHC. The general practitioners at the UHC ordered diagnostic tests (generally specific EBV serology and a full blood count [FBC]) when they saw a student with suspected IM. Eight students had diagnostic serology performed without a FBC, three had a FBC and rapid IM slide test for heterophile antibody without specific EBV serology, and three had diagnostic tests out with the UHC but belonged to the LCS. Of the 60 cases recruited, negative baseline serology was available for 28 who were already taking part in the LCS. Approximately 50% of all IM cases diagnosed at the UHC were recruited to the case control study. Reasons for non-participation included failure of the GP to give the student the study information leaflet, a student might also express an unwillingness to participate at this stage, return to the parental home to recuperate and a lack of telephone contact details.

Fifty eight seronegative students responded to a request by personal letter and acted as controls. The median age of the IM cases and controls was 20.1 years. (See Table 9).

	IM cases	Controls	p value
Number	60	58	
Male: Female	28:32	23:35	0.442
Median age years	20.1	20.1	0.981
Age Range years	18.2-27.3	18.6-23.8	

Table 9 Characteristics of IM cases and controls

Analysis by gender

Variables that showed significant differences by gender have been reported separately (fatigue duration, fatigue score, blood pressure, haemoglobin, gamma glutamyl transferase (GGT), and bilirubin).

7.2 Clinical Presentation and Symptoms

7.2.1 Presenting Symptoms

Students were asked which symptoms had caused them to seek medical attention. Usually students gave 3 symptoms (range 1-5). Sore throat was the commonest presenting symptom and was given as the reason for attending the GP by 47/60 (78%) Figure 4.

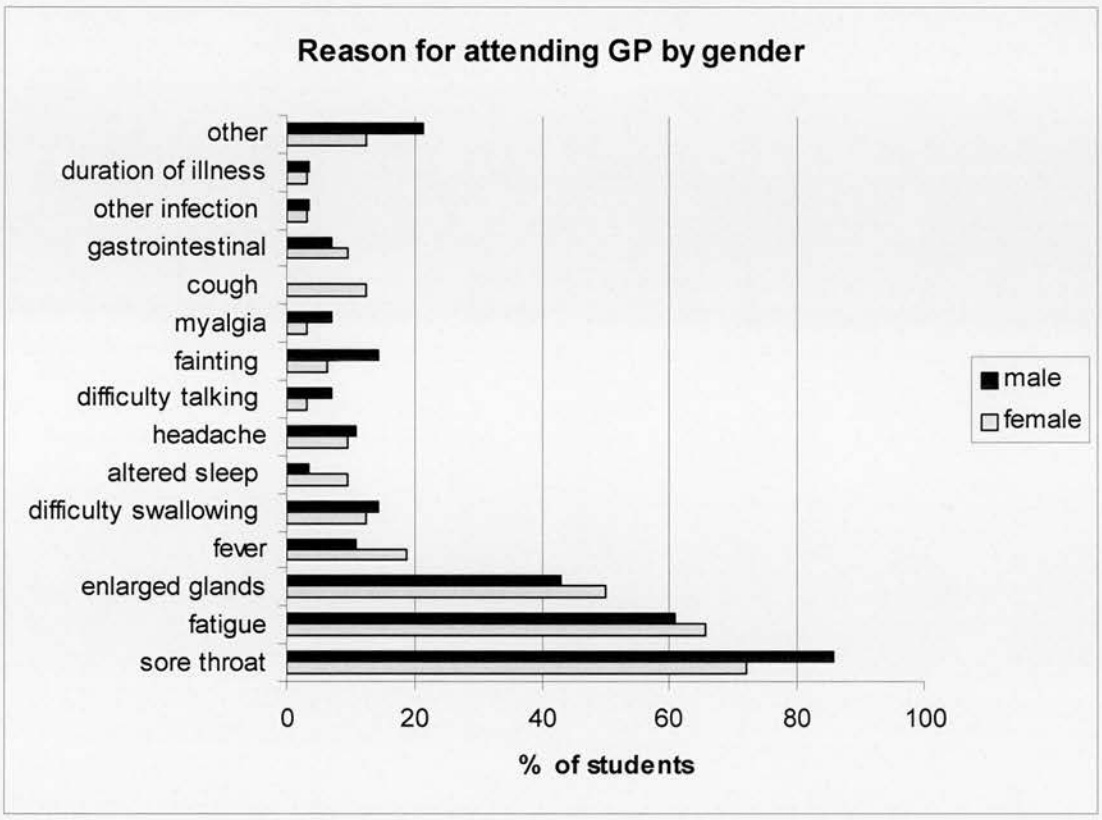


Figure 4 Presenting symptoms in IM

Tiredness, exhaustion or fatigue was the second most common presenting symptom if these terms were combined (38/60, 63%), followed by enlarged glands (28/60, 47%). Students were asked directly about the presence of sore throat, enlarged glands, fever, sweating, loss of appetite, headaches, pain in muscles or joints, and rash. Thus students had experienced additional symptoms without them being the precipitant for a visit to the GP. Asked in this way all but two of the 60 IM cases had experienced a sore throat as part of their illness, and all but two students had been aware of having enlarged glands (58/60, 97%). Forty percent of students (24/60) had to alter their diet because of their sore throat with 18/60 (30%) unable to swallow soft foods. One student, unable to swallow liquids or saliva, was admitted to hospital, another attended the casualty department and another was prescribed prednisolone by the GP. Students were asked about the duration of their severe symptoms

which lasted a median of 7 days for both genders. In the control group 6/58 (10%) had had a sore throat when seen, or in the preceding week, but none needed to modify their diet. Eight students (13%) developed a rash at some point during acute IM. In 6/8 cases (75%) this was not related to antibiotic therapy. Five students described urticarial rashes, and another student admitted to hospital with jaundice later developed a petechial rash. Two students developed rashes after antibiotics which had been prescribed by doctors out with the UHC. Only one of the two students with antibiotic-associated rash was able to recall his drug treatment which was sequential azithromycin, co-amoxiclav and penicillin. Forty seven of sixty (78%) students complained of reduced appetite, 46/60 (78%) thought that their temperature had been raised, although only one student had measured this, 45/60 (75%) had noticed sweating, 39/60 (65%) complained of headaches, and 33/60 (55%) had muscle or joint pains. Other symptoms elicited on direct questioning were fainting, near fainting or dizziness 11/60 (18%), altered sleep pattern 7/60 (12%), abdominal pain 7/60 (12%), nausea 7/60 (12%), vomiting 7/60 (12%), mouth ulcers 3/60 (5%) and a feeling of "vagueness", "muzziness" or being unable to concentrate 3/60 (5%). Four of the seven students (57%) that complained of nausea were taking antibiotics, whilst none of the students that complained of vomiting without nausea were so doing. Overall 14 students were prescribed antibiotics (23%). In two cases the students appeared to have an additional site of infection (dental abscess and lower respiratory tract infection). In four instances the antibiotic therapy was instigated by a medical practitioner out with the UHC.

7.2.2 Examination Findings

Of 45 students reviewed in the first month following diagnosis 10 (22%) had enlarged tonsils, and five (11%) had a tonsillar exudate. One "healthy" control was observed to have a tonsillar exudate presumably not related to EBV, as serology remained negative, and 6/58 (10%) had enlarged tonsils. Periorbital swelling was noted in two students with IM, but subjectively students' facial appearance had often appeared "slimmed down" at follow-up and therefore subtle facial swelling is probably common. The majority (53/60, 88%) of the IM cases had palpable lymphadenopathy at their first review, even if this visit occurred later than one month after diagnosis. This was significantly more often than the frequency of palpable nodes on the control group (23%, $p<0.001$). Amongst students with IM seen within a month of diagnosis 40/45 (89%) had palpable lymphadenopathy and eight (18%) had axillary as well as cervical lymphadenopathy. Of the 13 controls with palpable cervical lymphadenopathy two (4%) also had palpable axillary lymphadenopathy. Controls with palpable nodes were not more likely to have had a recent sore throat, however, a higher proportion showed evidence of seroconversion to anti-VCA IgG (7/13, 57%) compared to

those without lymphadenopathy (14/44, 32%, p=0.148). Of note, both controls with axillary lymphadenopathy had serological evidence of recent EBV infection with positive anti-VCA IgM. Amongst the IM cases, palpable splenomegaly was closely related to the time elapsed since diagnosis. Six of 57 IM cases (11%) had hepatosplenomegaly at their first visit, four (7%) had splenomegaly only, and 7 had isolated hepatomegaly (12%) (data was not recorded in three cases). All but one of the IM cases with splenomegaly or hepatosplenomegaly were seen within two weeks of diagnosis. In the students seen within two weeks of diagnosis, the proportion of students with hepatosplenomegaly, splenomegaly or isolated hepatomegaly was 11/26 (42%). In IM cases both splenomegaly (p=0.012) and hepatomegaly (p=0.022) were significantly associated with an elevated alanine transaminase. Seven of 52 controls (14%) had a palpable liver edge which was not considered pathological; (data were not recorded in six cases). Hepatomegaly in controls was not associated with abnormalities in liver function. Seven of 57 IM cases (12%, data not recorded in three) had isolated hepatomegaly, which is similar to the proportion seen in controls. None of the controls had splenomegaly. The pulse rate of the 45 IM cases seen within a month of diagnosis was greater (mean 74) than that of the control group (mean 69, p=0.02). No difference was detected when the systolic and diastolic pressures of cases and controls were compared, although both
diastolic and systolic reading were lower in female controls than males (see Table 10).

	Number	Mean	Range
Male systolic	23	129	97-148
Female systolic	35	116	89-137
p value		p<0.001	
Male diastolic	23	74	54-88
Female diastolic	35	70	56-86
p value		p=0.048	

Table 10 Blood pressure in male and female controls

7.2.3 Hospital Admission

Two male students were admitted to hospital as mentioned in the clinical presentation section 7.2.1 above. Briefly, one student developed jaundice, and the other was unable to

swallow fluids or saliva. A further student, also male, attended the accident and emergency department with symptoms of tonsillar swelling, and was prescribed prednisolone, but was not admitted.

7.3 Haematology Results

A full blood count was performed at each visit made by the IM cases and at the visit of each control student. Full blood counts were performed by the Department of Haematology, Royal Infirmary of Edinburgh, using an automated counter (SE-9000, Sysmex), at the same time as diagnostic EBV serology at the discretion of the GP. Forty seven of the 60 IM cases had a full blood count performed by the GP at the time of diagnosis. Normal ranges are those set by The Department of Haematology, Royal Infirmary of Edinburgh.

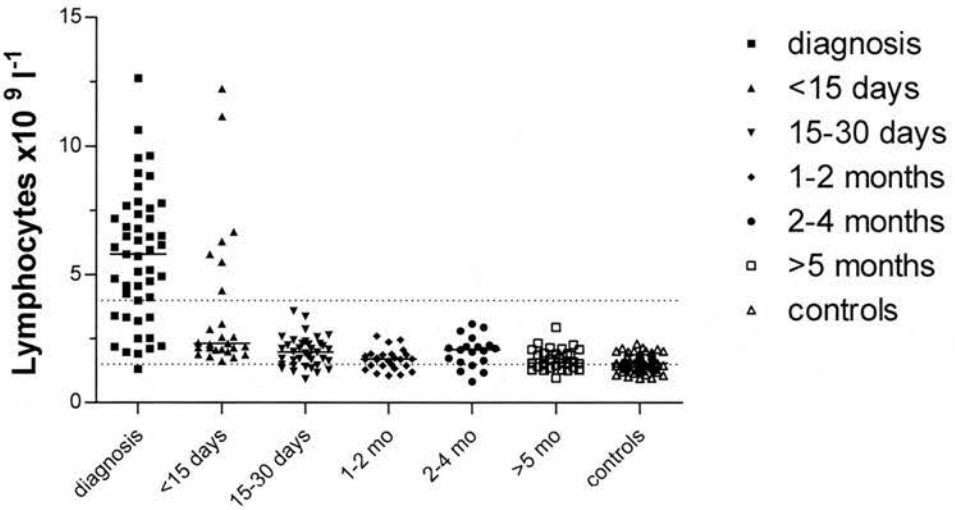
7.3.1 Lymphocyte Counts

Forty seven students had a lymphocyte count performed at the time of diagnosis. In 35 cases (75%) the result exceeded the upper limit of the normal range (1.5-4.0 $x10^9$ l^{-1}) and was below the lower limit in one. Thus 23% of students had a lymphocyte count in the normal range at the time of diagnosis. (See Table 11). None of the controls had a lymphocytosis; almost half (46%) were classified as being lymphopenic, according to the normal range set by the haematology laboratory, although only two had lymphocyte counts less than 1.0 $x10^9l^{-1}$. The timing of the full blood count in IM cases with normal lymphocyte counts was not significantly different from students with abnormal results with respect to the timing of their most severe symptoms (which had occurred a median of four days previously and 6.5 days previously respectively). The lymphocyte count of the IM group significantly exceeded that of the control group in the first month after diagnosis and again in cases reviewed between 2 and 4 months after IM (See Figure 5).

Time since diagnosis	Diagnosis	<15 days	15-30 days	1-2 months	2-4 months	≥ 5 months	Control
Number of cases	47	26	37	26	19	35	54
Median count							
Lymphocytes x 10^9 l^{-1}	5.8 p<0.001	2.3 p<0.001	2.0 p<0.001	1.7 p=0.22	2.1 p=0.001	1.6 p=0.09	1.6
Monocytes x 10^9 l^{-1}	1.4 p<0.001	0.5 p=0.032	0.4 p=0.86	0.4 p=0.11	0.5 p=0.26	0.4 p=0.27	0.4
Neutrophils x 10^9 l^{-1}	2.8 p=0.015	2.7 p<0.001	2.6 p=0.001	2.4 p<0.001	3.0 p=0.30	3.1 p=0.57	3.3
Platelets x 10^9 l^{-1}	188 p<0.001	258 p=0.70	235 p=0.09	235 p=0.12	242 p=0.35	222 p=0.045	252
Hb g l^{-1} (male)	(n=23) 142.0 p=0.004	(n=8) 141.0 p=0.002	(n=16) 140.5 p<0.001	(n=9) 147.0 p=0.10	(n=7) 154.0 p=1.0	(n=15) 153.0 p=0.58	(n=22) 154.0
Hb g l^{-1} (female)	(n=24) 131.5 p=0.68	(n=18) 129.5 p=0.31	(n=21) 127.0 p=0.052	(n=17) 127.0 p=0.079	(n=12) 129.0 p=0.24	(n=20) 130.5 p=0.53	(n=32) 132.5

g gram l litre

Table 11 Haematology parameters in IM cases at diagnosis, during convalescence and in controls

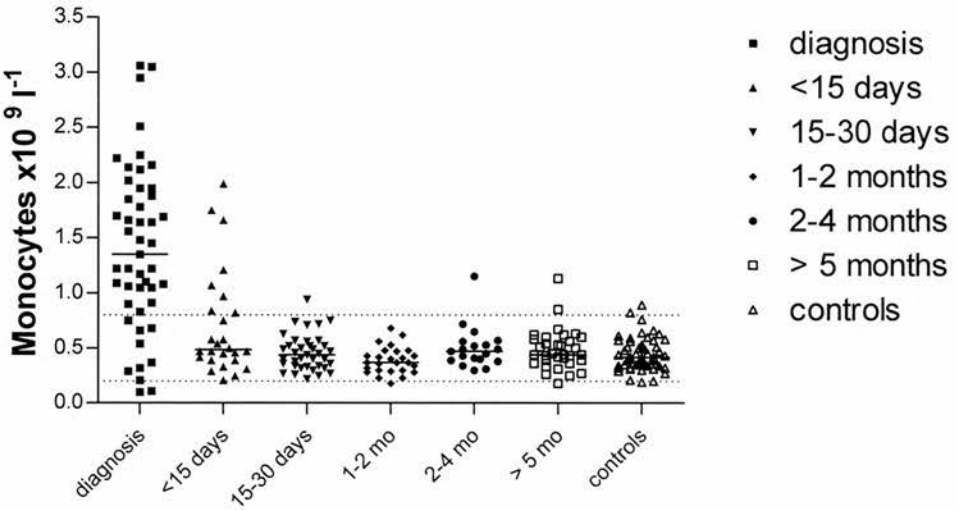


The normal range (1.5-4.0 x10^9 l^{-1}) is shown by the faint dotted line

Figure 5 Total lymphocyte count in IM at diagnosis, during convalescence and in controls

7.3.2 Monocyte Counts

At diagnosis 37/47 (79%) of students had an elevated monocyte count (>0.8 x10^9 l^{-1}) and 2 (4%) had a reduced count (<0.2 x10^9 l^{-1}). The median monocyte counts at the time of diagnosis and for students with blood collected within 15 days of diagnosis were significantly greater than that of the control group median ($p<0.001$ and $p=0.032$ respectively) as shown in Table 11 and Figure 6. Monocyte counts later in the clinical course were not significantly different from control values.

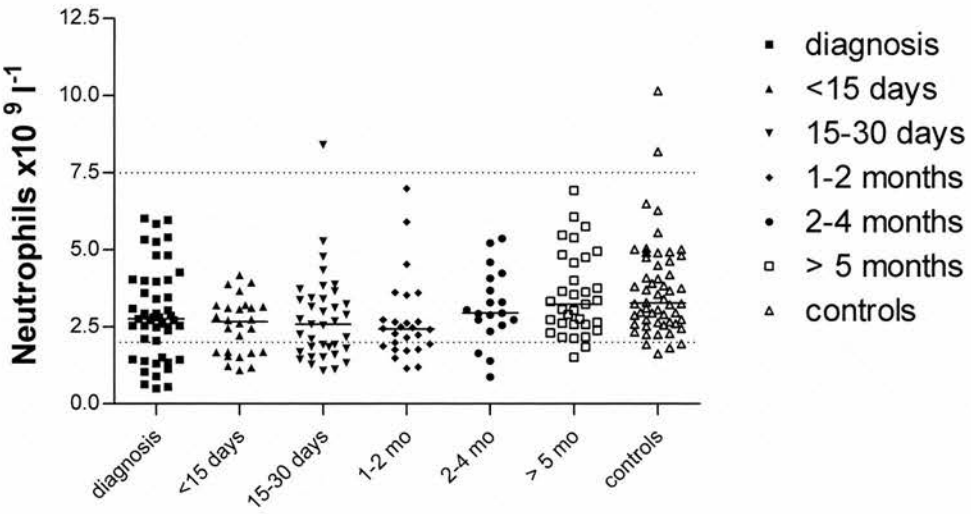


The normal range (0.2-0.8 x10^9 l^{-1}) is shown by the faint dotted line

Figure 6 Monocyte count in IM at diagnosis, during convalescence and in controls

7.3.3 Neutrophil Counts

At the time of diagnosis the neutrophil count in IM cases was reduced (median 2.8 x10^9 l^{-1}) when compared with healthy controls (median 3.3 x10^9 l^{-1}, p=0.015) (Table 11). Twelve of 47 IM cases (26%) had a neutrophil count below the lower limit of the normal range (2.0-7.5 x10^9 l^{-1}) at diagnosis and in 4 (9%) the neutrophil count was less than 1.0 x10^9 l^{-1}. None had an elevated neutrophil count at the time of diagnosis. In IM cases the neutrophil count shows a statistically significant increase with time elapsed since diagnosis, (Spearman correlation p=0.035). The neutrophil counts of IM cases remained significantly lower than that of controls until 2 months after diagnosis, as shown in Table 11 and Figure 7. Male control students had a lower median neutrophil count (2.94 x10^9 l^{-1}) than female controls (median 3.62 x10^9 l^{-1}), although this did not attain statistical significance, (p=0.09).

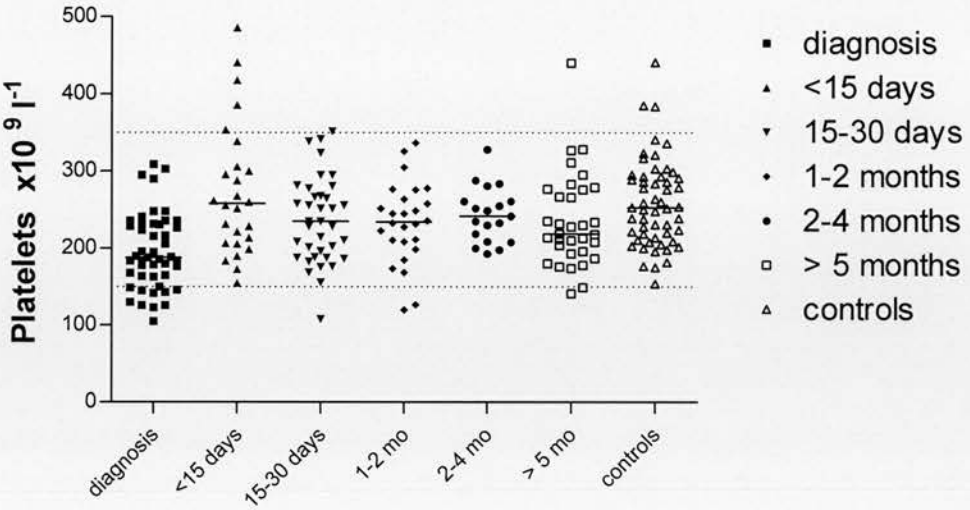


The normal range (2.0-7.5 x10^{9} l^{-1}) is shown by the faint dotted line

Figure 7 Neutrophil count in IM at diagnosis, during convalescence and in controls

7.3.4 Platelet Counts

Platelet counts were significantly reduced at diagnosis when compared with controls (IM median 188 x $10^{9}l^{-1}$, control median 252 x $10^{9}l^{-1}$, $p<0.001$) as shown in Table 11 and Figure 8). Ten of 47 IM cases (21%) had a platelet count below the normal range (150-350 x10^{9} l^{-1}) at the time of diagnosis. None were less than 100 x 10^{9} l^{-1}, and all were asymptomatic. All but one of 43 retested with a month had a platelet count above the lower limit of the normal range. Platelet counts of convalescent students did not differ significantly from those of the controls (except the late follow-up group (≥5 months) where the median platelet count in the control group was greater ($p=0.045$)).



The normal range (150-350 x10^{9} l^{-1}) is shown by the faint dotted line

Figure 8 Platelet count in IM at diagnosis, during convalescence and in controls

7.3.5 Haemoglobin

Haemoglobin values differed between males and females and were therefore analysed separately as shown in Table 11. In male IM cases haemoglobin values at diagnosis (median 142 g l^{-1}) were significantly lower than those of the male control group (median 154 g l^{-1}, p=0.004). Significantly lower values were seen in male cases compared with controls for one month after diagnosis. In female IM cases median haemoglobin values were lower than those of the female control group (control group median 132.5 g l^{-1}) at all time points. These results bordered on significance for the groups seen between 15 and 30 days after diagnosis (127 g l^{-1}, p=0.052) and 1 to 2 months (127 g l^{-1} , p=0.079) after IM.

7.4 Liver Function Tests

Liver function tests (alanine transaminase (ALT), alkaline phosphatase (ALKP), gamma glutamyl transferase (GGT) and bilirubin) were measured using an automated analyser (Olympus AU640, Watford) by the Department of Clinical Biochemistry, Edinburgh Royal Infirmary, and compared to the normal range set by that Department.

LFTs are not routinely performed at diagnosis by general practitioners at the UHC. Seven students (2 male, 5 female) were incidentally found to have had these performed. The

reasons that they were performed are not known and therefore some clinical bias is possible. The LFTs were performed by 4 GPs from the 8 doctor practice. Students that joined the case control study had LFT performed at their first study visit. Students with abnormal results had their LFTs repeated at follow-up visits until their results (except bilirubin) returned to the normal range. Control students had LFTs performed once. Two control students (one with polycystic renal disease) were found to have LFT results out with the "normal range" provided by Edinburgh Royal Infirmary. No gender difference in LFT was observed for ALT or ALKP. Bilirubin and GGT differed by gender and were analysed separately.

Time since diagnosis	Diagnosis	<15 days	15-30 days	1-2 months	2-4 months	≥ 5 months	Control
Number of cases	7	25	18	7	4	4	53
Median count							
ALT $U\,l^{-1}$	135 p<0.001	74 p<0.001	32 p<0.001	14 p=0.32	20 p=0.28	16 p=0.69	16
ALKP $U\,l^{-1}$	103 p=0.002	99 p<0.001	83 p=0.02	65 p=0.87	59 p=0.54	71 p=0.64	69
GGT (male) $U\,l^{-1}$	(n=2) 90 p=0.042	(n=9) 39 p<0.001	(n=12) 31 p<0.001	(n=2) 14 p=0.78	(n=2) 44 p=0.15	(n=3) 18 p=0.43	(n=21) 14
GTT (female) $U\,l^{-1}$	(n=5) 50 p=0.040	(n=16) 39 p<0.001	(n=6) 22 p=0.013	(n=5) 12 p=0.89	(n=2) 18 p=0.06	(n=1) 10 insufficient data	(n=32) 12
Bilirubin (male) $\mu mol\ l^{-1}$	(n=2) 20 p=0.33	(n=9) 10 p=0.036 but < controls	(n=12) 17 p=0.74	(n=2) 14 p=0.55	(n=2) 15 p=1.0	(n=3) 13 p=0.73	(n=21) 16
Bilirubin (female) $\mu mol\ l^{-1}$	(n=5) 7 p=0.18	(n=16) 8 p=0.12	(n=6) 13 p=0.011	(n=5) 10 p=0.86	(n=2) 13 p=0.63	(n=1) 12 insufficient data	(n=32) 9

U unit l litre

Table 12 Liver function tests at visit one by time elapsed since diagnosis in IM cases, and controls

7.4.1 Alanine Transaminase

IM cases that had LFT performed within in one month of diagnosis had significantly raised ALT values compared with control students as shown in Table 12. Eight of twelve (67%) cases with hepatomegaly had a raised ALT compared with 13 of 43 (30%) cases that did not have a palpable liver (p=0.022). None of the control students had an elevated ALT. Of 27 cases with ALT performed in the first two weeks of illness 24/27 (89%) were raised, 41% were more than twice the upper limit of normal and 19% were more 5 times the upper limit of normal.

7.4.2 Alkaline Phosphatase

ALKP values in students with IM were significantly elevated when compared to control values in the first month after diagnosis as shown in Table 12. ALKP was less likely to be elevated in acute IM than ALT. Of 27 cases with ALKP performed in the first two weeks of illness 5/27 (19%) were raised, and all students with an abnormal ALKP also had a raised ALT. Of students reviewed in the first month after diagnosis 6/7 (86%) with a raised ALKP had a palpable liver whereas only 1/31 (3%) without a hepatomegaly had an abnormal ALKP (p<0.001). None of the controls had an elevated ALKP.

7.4.3 Gamma Glutamyl Transferase

The normal range of GGT values given by Edinburgh Royal Infirmary is lower in females, than males, which was affirmed by analysis of GGT values obtained from male and female controls enrolled in the study. The median value in males (14 U l^{-1}) was significantly higher than that in females (12 U l^{-1}, p=0.023). An elevated GGT was found in 16/58 (28%) IM cases at their first visit and in 2/53 (4%) controls but was not found to be a significant marker of organomegaly in either group, (see Table 12).

7.4.4 Bilirubin

Bilirubin values in male controls exceeded those of female controls. The "normal" range is given as 2-17 μmol l^{-1} for both sexes by Edinburgh Royal Infirmary, however, the results have been analysed by gender in view of the significantly greater values for bilirubin in male controls (median 16 μmol l^{-1}) than female controls (median 9 μmol l^{-1}, p<0.001) (Table 12). Elevation of bilirubin was not significantly related to enlargement of the liver or spleen in cases or controls.

7.5 Antibody to Epstein-Barr Viral Capsid Antigen and Heterophile Antibody

Serological tests for IgG and IgM antibodies to VCA and heterophile antibody were

performed on plasma from all controls (n=58), and from IM cases (n=60) at diagnosis where a sample was submitted by the GP (n=52) and at subsequent follow up visits. The diagnosis of IM was based on the presence of anti-VCA IgM, or, the presence of a positive heterophile antibody test and the demonstration of anti-VCA IgG seroconversion.

7.5.1 Heterophile Antibody

Heterophile antibody was detected in 51/52 (98%) cases where a diagnostic sample was sent by the GP. The IM case with the initially negative heterophile result had a positive result when reviewed after 3 weeks. IM cases reviewed within 2 weeks of diagnosis had a positive result in 28/29 (97%), at four weeks this had fallen to 89% (32/36), at 1-2 months 69% (18/26), 47% (9/19) at 2-4 months and 22% (7/32) in those seen after 5 months still had a positive result (Figure 9). Two of the 58 control students (3%) had a positive heterophile test. Both had undergone IgG seroconversion and one had a (weakly) positive IgM result.

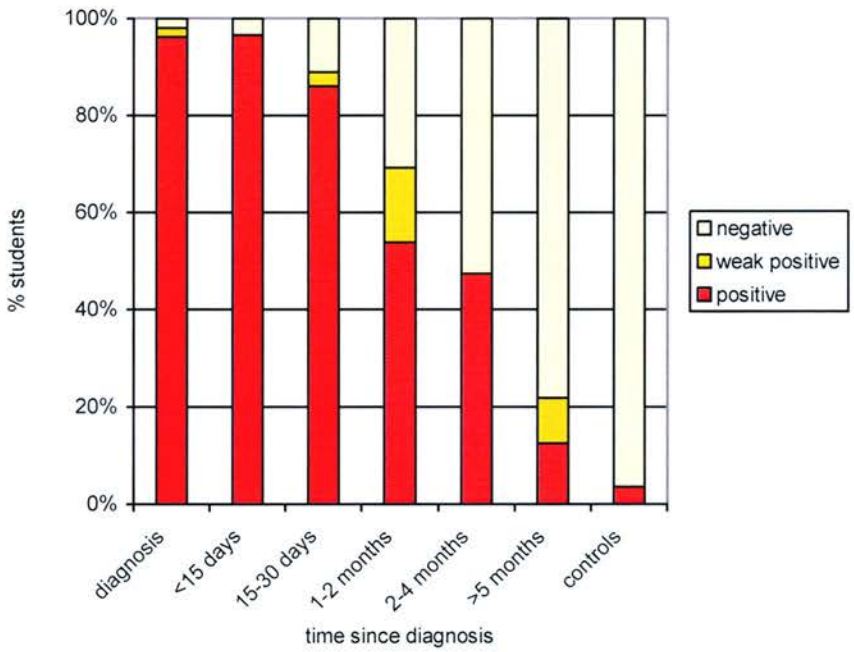


Figure 9 Heterophile antibody in IM at diagnosis, during convalescence and in controls

7.5.2 IgM Antibodies to Viral Capsid Antigen

IgM antibodies to VCA were detected in 48/52 (92%) of the IM cases where a diagnostic sample was sent by the GP. Amongst the 46 IM cases reviewed within one month of their initial diagnosis 45 (98%) had anti-VCA IgM. Two IM cases never showed a positive anti-

VCA IgM test. Anti-VCA IgM antibodies declined over time from diagnosis with 69% (18/26) positive at 1-2 months, 37% (7/19) at 2-4 months and 13% (4/32) after 5 months. Three of 58 (5%) controls had detectable anti-VCA IgM; all had seroconverted to anti-VCA IgG and one also had a positive heterophile antibody test (Figure 10).

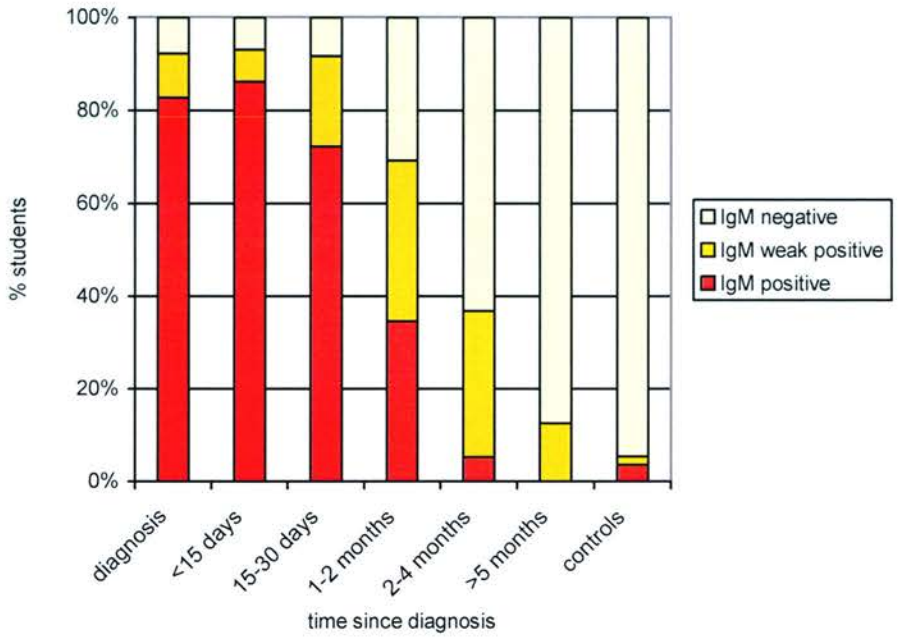


Figure 10 Anti-VCA IgM in IM at diagnosis, during convalescence and in controls

7.5.3 IgG Antibodies to Viral Capsid Antigen

IgG anti-VCA antibodies were detected in 51/52 (98%) samples submitted at diagnosis of IM. The IM case with no detectable anti-VCA IgG at diagnosis did have detectable anti-VCA IgG when reviewed 8 days later. Antibody titres in IM cases varied throughout the study with a tendency to fall during the convalescent period (Figure 11). However in 6 cases with low or absent (1 case) anti-VCA IgG at diagnosis; increased titres were seen in later samples. At diagnosis 29% (15/52) of IM samples showed antibody levels above the test normal range (<1:640), and this figure reduced to 26% (5/19) at follow-up between 2 and 4 months, and 19% (6/32) at follow-up 5 or more months after diagnosis. Twenty two of the 58 controls (38%) had detectable anti-VCA IgG indicating silent seroconversion, and of these one had detectable IgM and heterophile antibody, 2 had anti-VCA IgM alone, and 1 had a positive heterophile test alone.

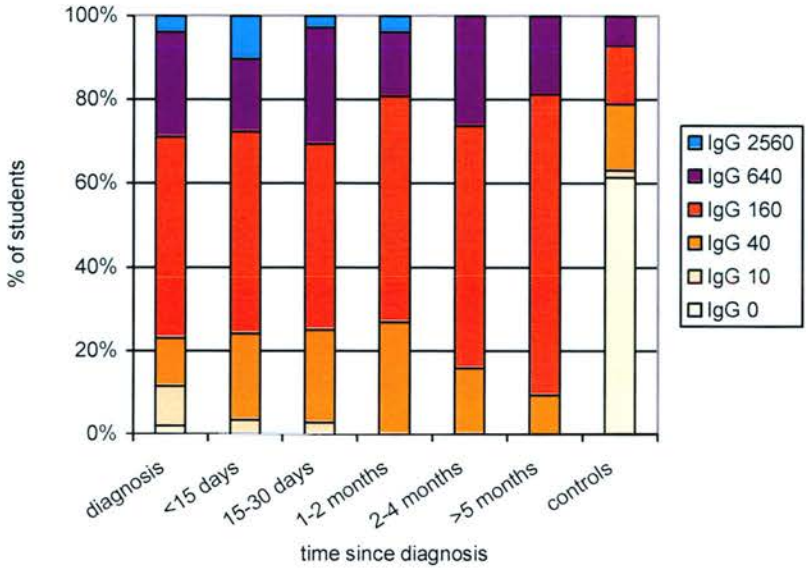


Figure 11 Anti-VCA IgG in IM at diagnosis, during convalescence and in controls

7.6 Viral Load

The viral load was measured by a semi quantitative technique, by Dr. K. McAulay (University of Edinburgh), on 46/60 (77%) students at the time of diagnosis (no diagnostic sample in eight, no stored DNA in 4 early samples and insufficient in two). Viral load quantification at follow-up visits was performed on 133/150 (89%) occasions. There was a highly significant reduction in viral load with days elapsed since diagnosis ($p<0.001$)(see Figure 12).

Overall, at the time of the diagnostic sample students had been unwell for a median of 5 days (maximum 46 days). Four students complaining of vomiting had a significantly higher viral load (median, 72, 225 genomes per 10^6 cells) than those without vomiting (5807, $p=0.005$). The association between the presence of other symptoms and the viral load at diagnosis was investigated. Students with some types of symptoms consistent with a more severe illness (e.g. fever, sweating, myalgia, severe sore throat resulting in modified diet, reduced walking distance) did often have a higher median viral load at diagnosis, but these did not attain statistical significance in the numbers of students studied (Table 13). Students suffering from persistent fatigue at six months after diagnosis also had a higher median viral load at diagnosis (12, 400 genomes per 10^6 cells) than students that had made a complete recovery

by this time (5773 genomes per 10^6 cells) but this did not attain statistical significance, nor was the viral load at diagnosis correlated with the duration of fatigue in males or females. Fatigue is discussed in more detail in section 7.8.9. No significant correlation between the viral load at diagnosis and duration of severe symptoms, or the initial total white cell count, lymphocyte count, neutrophil count, monocyte count or platelet count was found.

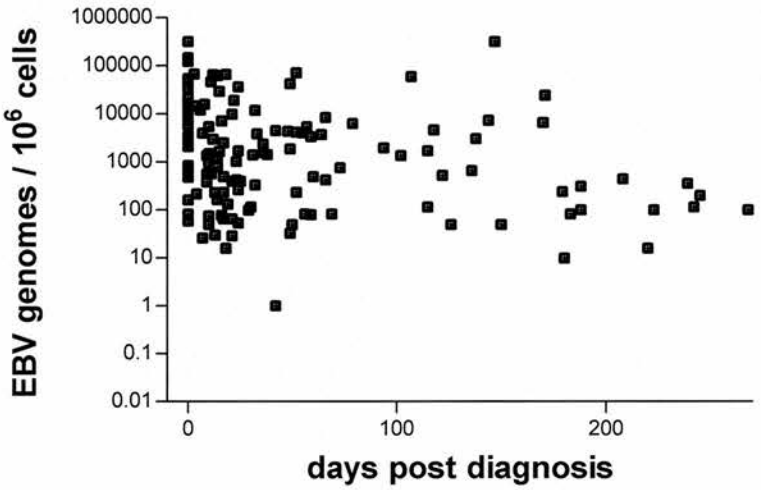


Figure 12 EBV viral load in IM cases vs. days since diagnosis

	Number of students with variable	Median VL at diagnosis if variable present (Range) copies per 10^6 cells	Median VL at diagnosis if variable absent (Range) copies per 10^6 cells	p
Fever	37/46	7800 (0-318300)	7366 (82-147850)	0.890
Sweating	36/46	8283 (0-318300)	5201 (82-147850)	0.842
Muscle aches	28/46	8825 (0-417850)	4636 (161-318300)	0.492
Headache	29/46	3500 (0-147850)	10,466 (161-318300)	0.387
Anorexia	36/46	6604 (0-318300)	8870 (161-33783)	1.0
Nausea	6/46	2563 (59-16840)	8283 (0-318300)	0.463
Vomiting	4/46	72225 (16840-318300)	5807 (0-147850)	p=0.005
Rash	7/46	2673 (59-33783)	8766 (0-318,300)	0.275
Unable to follow usual diet	19/46	8883 (0-54300)	3036 (0-318300)	0.446
Unable to tolerate soft diet	16/46	12,840 (0-54300)	5807 (0-318300)	0.423
Unable to leave home during worst symptoms	19/46	8766 (82-147850)	5841 (0-318300)	0.076
Walking distance <100m at worst	24/46	9692 (82-147850)	2855 (0-318300)	0.063
Fatigue at 6 months	9/40	12,400 (0-48283)	5773 (0-318300)	0.391
Part-time term employment	9/43	8883 (82-54300)	6604 (0-318300)	0.870
Impaired concentration	38/46	8283 (0-318300)	4933 (483-16166)	0.460

Table 13 Clinical features of IM cases and viral load at diagnosis

7.7 EBV Strain Typing

7.7.1 EBV Strain Typing (type 1 or 2) in Students with IM

In order to determine the relative contribution of type 1 and 2 strains to disease presentation EBV typing was carried out using PCR which amplified type specific sequences within the EBNA3C gene (Brooks *et al*, 2000; Sample *et al*, 1990). DNA was successfully amplified in 48/53 samples (91%). Forty two isolates were type 1 strains (88%), 3 were type 2 (6%) and a further 3 (6%) contained both type 1 and type 2 sequences.

7.7.2 EBV type and Lymphocyte Count at Diagnosis

The number of IM cases caused by type 2 strains was insufficient to permit investigation of

any associations between EBV type and clinical symptoms, laboratory parameters or viral load. Lymphocyte counts and EBV type were made available to me for an additional ten IM cases by Drs. R. Thomas and K. McAulay. Interestingly IM caused by EBV type 1 may be associated with a higher lymphocyte count in the peripheral blood (see Appendix 12.2).

7.7.3 Strain Typing of IM Cases and their Contacts

In order to compare EBV isolates obtained from IM cases and their contacts strain typing was performed by examining the numbers of repeats in the EBNA 3C and LMP1 regions of the EBV genome; two regions known to carry variable numbers of repeat sequences. (see 5.10.3).

Samples obtained

Mouth gargle samples and or peripheral blood samples were collected from the partners, friends and flatmates of students with IM. Overall 61 contact pairs were identified. The largest group being flatmates (31) followed by partners (20), friends (10), IM cases known to each other as friends or flatmates (4 pairs) and a sister, who had also contracted IM. The samples collected were venous blood and throat gargle (31), capillary blood and throat gargle (1), venous blood alone (8) and mouth wash alone (12). Among the 40 contacts with serum or plasma available, 35 (88%) were EBV seropositive. Sixteen of the mouthwash samples were collected in the students' own homes supervised by the student who had contracted IM. These samples were subject to greater delays in processing and subjectively the sample quality of some of the throat gargles looked poor, however, the likelihood of obtaining a typing result was not significantly less than that achieved with the supervised gargles (31% vs. 44% p=0.50).

Results

If amplification at both loci was successful a discrepancy at one was interpreted as a mismatch. If, however, only one locus amplified and this matched; this was termed a "partial match". In 11 partner pairs where amplification was successful, 6 matched at both loci, 3 showed partial match and 2 were non-identical (18%).

In the non-sexual contacts there were 2 pairs with matches at both loci, 1 partial match, and 14 mis-matched pairs (82%). Thus sexual contacts were significantly more likely than non-sexual contacts to share strains (p=0.001).

7.8 Impact of IM on Studies and Other Activities

7.8.1 Activities of Daily Living

Students were asked if they were able to get up, dress and wash during the worst phase in their illness. Whilst this might be affected by important study commitments 50/58 (86%) could wash and dress during their worst symptoms. Data were not recorded by two students. Students were asked if they were able to leave their home at the worst stage of their illness. This figure was lower with only 36 of 59 (61%) of students stating that they left home during their worst symptoms. A related question was how far students were able to walk. Amongst students with IM that were able to leave their home 23/35 (66%) could not walk as far as normal. Five students stated that they were unable to walk 100 m and a further 9 estimated that they were unable to walk 500 m. All but one student stated that they would normally be able to walk over 2 km (or for longer than 30 minutes at a normal pace). Of the 56 IM cases completing this question only 12 (21%) were able to walk over 2 km during the acute phase of their illness.

7.8.2 Hours of Sleep

Hypersomnia is a recognised feature of IM. The reported hours of sleep in cases exceeded that of controls. This difference was still apparent at review five or more months after diagnosis when cases continued to sleep for an hour longer than the control group (Table 14). Unfortunately data on hours of sleep prior to the onset of IM did not form part of the survey although this information was available for 9 students who developed IM. Their pre-morbid hours of sleep did not differ from that of the controls (mean 7.4 hours for cases and 7.3 for controls) suggesting that the IM group did not differ from the control group prior to the onset of their illness.

In addition students with IM reported an increase in hours spent lying down but not asleep. Students reviewed within the first two weeks of diagnosis spent a median of two hours per day spent lying down but not sleeping compared with one hour for the control group ($p=0.001$) (Table 14).

Time since diagnosis	Diagnosis	<15 days	15-30 days	1-2 months	2-4 months	≥ 5 months	Control
Number of cases	n=60	n=31	n=37	n=27	n=20	n=34	n=58
Activity							
Studying †	0 [n=44] p<0.001	15 [n=27] p=0.001	22.3 [n=34] p=0.119	24.5 [n=24] p=0.321	24.5 [n=16] p=0.346	26 [n=27] p=0.873	26 [n=56]
Exercising	0 [n=59] p<0.001	0 p<0.001	0 [n=29] p<0.001	1 [n=20] p=0.038	2 p=0.945	2 p=0.864	2
Social activities	0 p<0.001	4 p<0.001	5 p<0.001	6 p=0.004	10 p=0.220	8 [n=33] p=0.004	12
Sleeping ‡	-	10 p<0.001	9 [n=36] p<0.001	8 p=0.002	8.5 [n=19] p<0.001	8 p<0.001	7 [n=57]
Resting not sleeping	-	2 [n=27] p=0.001	1 [n=35] p=0.093	1 [n=25] p=0.525	1 p=0.864	1 p=0.371	1 -

Values expressed as median hours per week, except hours of sleep‡ / 24 hours

† Sum of hours of private study and timetabled classes per week

Table 14 Hours spent per week on different activities in IM at diagnosis, during convalescence and in controls

7.8.3 Concentration

The majority (47/60, 78%) of students thought that IM had affected their ability to concentrate (see "Physical Function and Fatigue" Appendix 12.1). This perception did not vary with gender, but was significantly influenced by whether the worst symptoms had occurred during the holidays. Of the thirteen students who stated that their concentration had not been affected, six (46%) were on holiday at the time of their most severe symptoms, whereas, amongst the students stating that their ability to concentrate was impaired, only 15% (7/47) were on vacation at the time of their worst symptoms. This difference was

statistically significant (p=0.015).

Students that stated their ability to concentrate was impaired estimated the period of time they could learn new information, either in a lecture or from a textbook. At the time of their most severe symptoms this was ten minutes or less for 70% (31/44) of the students reporting impaired concentration. This information was collected in 25 of the control group of whom 22/25 (88%) could concentrate for 40 minutes or more. When compared with control students the IM cases reported a significantly reduced concentration span for one month after diagnosis.

7.8.4 Hours Attending University

Cases, prior to IM, and controls reported spending similar numbers of hours attending university (Table 15). Amongst controls no significant gender difference in attendance was detected.

	Cases (before IM)	Controls	p value
Number	59	58	
Attending university	16 (0†-48) $^{n=59}$	15 (6-40)	0.935
Private study	10 (2-60) $^{n=58}$	10 (0-50) $^{n=56}$	0.312
Total study hours	27 (5-66) $^{n=58}$	26 (8-80) $^{n=56}$	0.306
Social activities	12 (3-25)	12 (3-41)	0.571
Exercise	4 (0-20)	2 (0-12)	p=0.008

Median hours per week, range in parentheses; † two PhD students gave University hrs=0

Table 15 Reported weekly hours of study, exercise and social activities in cases prior to IM and in controls

As expected the development of IM had an adverse effect on students' attendance at university. An estimation was made at each interview of the actual timetabled hours of university sessions that the student had been unable to attend on account of IM. Overall the median number of lost timetabled hours was ten, with a range of none to 300. Seventeen of 60 (28%) lost no university time, although 13 of these 17 were on holiday at the time of their most severe symptoms. Twenty two (37%) students lost up to 20 hours and six (10%) missed over 100 hours. Female students reported missing significantly more university classes than male students, a median of 17 vs. 2 hours, (p=0.004) (Table 16). The occurrence of a student's most severe symptoms during a holiday period offered significant protection against missing timetabled university classes (median hours missed if onset during

vacation 0, onset during term median 15 hours, p=0.001). Onset of illness during a vacation completely protected male students (7/7) from missing timetabled classes whereas 3/6 female students still missed university time (4, 59 and 126 hours). The five students, all female, that either left university completely or decided to repeat the year missed between 36 and 300 hours (average 115, median 72).

	Number	Median	Range
Male	28	2	0-80
Female	32	17	0-300
p value		p=0.004	

Table 16 Hours of timetabled university classes missed through illness in male and female IM cases

The severity of initial disability predicted the quantity of university classes missed. Students with IM that could not wash and dress at the worst point of their illness, or that were unable to leave their house, or reported a reduced walking distance all missed a significantly greater number of timetabled classes than cases who did not report these symptoms (Table 17).

	Number of students with variable	Median hours of missed timetabled classes if variable present (Range)	Median hours of missed timetabled classes if variable absent (Range)	p value
Unable to wash and dress during worst symptoms	8/57	56 (0-300)	7 (0-126)	p=0.009
Males	3/27	39 (20-40)	1 (0-80)	p=0.039
Females	5/30	115 (0-300)	15 (0-126)	0.066
Unable to leave home during worst symptoms	23/58	35 (0-300)	3 (0-126)	p=0.004
Males	8/27	15.5 (0-80)	0 (0-70)	0.182
females	15/31	54 (0-300)	10 (0-126)	p=0.030
Reduced walking distance during worst symptoms	48/59	15.5 (0-300)	0 (0-46)	p=0.005
Males	20/27	4 (0-80)	0 (0-46)	0.156
Females	28/32	35 (0-300)	4.5 (0-11)	p=0.035
Walking distance <100m during worst symptoms	28/58	18.5 (0-300)	2.5 (0-126)	p=0.005
Males	10/27	15.5 (0-80)	0 (0-70)	0.078
Females	18/31	44 (0-300)	11 (0-126)	0.128

m meters

Table 17 Severity of initial disability in IM cases and subsequent number of missed timetabled classes

At interview there was an impression that students studying science subjects missed a greater number of timetabled university hours, presumably reflecting the significantly greater number of timetabled hours in their courses overall, however this did not attain statistical significance (see Table 18).

	n	Timetabled hours per week	Median hours of missed university classes	Range (hours) missed timetabled university classes
Science, medicine, veterinary studies	28	20.0	14.5	0-300
Social sciences	16	13.0*	8.0	0-126
Arts courses	15	11.5†	5.0	0-115
p value		* $p<0.001$ † $p<0.001$	all NS	

NS not significant n Number of students

Table 18 Timetabled university hours missed through illness by degree type

7.8.5 Hours of Private Study

Students were asked at each visit if their illness was affecting their ability to study. Over time there was a gradual increase in the proportion of students returning to their pre-illness study routines (Table 19). At the time of diagnosis reduced private study was almost universal with only 8% of students able to follow their normal study routine. Counting the hours of timetabled university classes missed through illness is reasonably objective, however, estimating the hours that might have been spent in private study, but were not because of illness, is much more difficult.

Time since diagnosis	Study routine disrupted	Study routine not affected	*	% following usual study routine
During worst symptoms	47	4	9	8
≤ 15 days	27	4	0	13
15-30 days	22	12	3	35
1-2 months	7	18	2	72
2-4 months	3	15	4	83
≥ 5 months	4	27	5	87

*left university or on vacation

Table 19 Proportion of students with normal study routine by time after IM diagnosis

In addition, for some students the distinction between attending university and private study was not clear-cut. Three of the students with IM were PhD students, another was writing a Master in Law and at least three others were undertaking science honours dissertations when they contracted glandular fever. Furthermore during exam time students may spend many hours in private study but none in timetabled classes. Hours of private study and the total hours of study (timetabled classes and private study) in cases prior to IM and in controls are shown in Table 15.

When cases were compared with control students their total hours of study were significantly reduced for two weeks after diagnosis (see Table 14).

Since the median duration of severe symptoms was 7 days (see 7.2.1) and study time was significantly reduced compared with controls for a further 2 weeks (Table 14) an estimation of the total study hours lost through illness can be made by comparing the reported behaviours of cases and controls. During the first seven days cases reported that they undertook no university work at all, whilst controls completed 26 hours. During the following two weeks cases completed 15 hours (median) per week (11 hours less than the control students). Thus a very crude estimation of 48 hours of combined university classes and private study time might be lost for students contacting IM during term time.

7.8.6 Repeat Academic Year / Left University

Five students in this case control study (8%) were known to have discontinued their course after contracting IM. Two left university completely and three planned to repeat the

academic year during which they had contracted IM.

7.8.7 Social Activities

Cases when well and controls spent similar amounts of time attending social events (e.g. pub, club, cinema, going out for a meal, see 12.1) (Table 15). There was no significant gender difference in the number of hours spent on social activities in control subjects or in cases prior to the onset of symptoms. After contracting IM cases spent fewer hours on social activities than controls at all periods during convalescence, and this was significant for all time periods except for students reviewed between 2 and 4 months. As might be anticipated the reduction was most marked in the early weeks after diagnosis (see Table 14).

7.8.8 Exercise

Six of 60 IM cases (10%) and 15/58 (26%) controls undertook no vigorous exercise (defined as getting out of breath e.g. running, cycling, exercise class, swimming, see 12.1) ($p=0.222$). Unlike the number of hours that students spent studying or socialising, which did not differ between cases and controls, the reported hours spent undertaking vigorous exercise (prior to the onset of IM) was greater in cases (median 4 hours per week) than in control (median 2 hours, $p=0.008$) (Table 15). There was no significant difference in the number of hours of exercise per week reported by male (median 3 hours) and female controls (median 2 hours, $p=0.274$) or between male (median 3.5 hours) and female IM cases (median 4 hours, $p=0.988$) prior to the onset of IM.

In the acute phase of their illness 56/60 (93%) of cases had reduced or ceased their usual exercise regime, and even 5 months after diagnosis one quarter of cases were taking less exercise than prior to diagnosis. Comparison with controls shows that cases took significantly less exercise for up to 2 months following diagnosis (Table 14), but this comparison is problematic in that controls reported fewer hours of exercise than cases prior to the onset of IM as discussed above.

7.8.9 Fatigue Score and Duration

Students were asked to rate their feeling of fatigue on a numerical rating scale from zero (no fatigue) to 10 (as bad as you can imagine). Students reviewed within one month of diagnosis and students reviewed later did not differ in their rating of fatigue before they had become unwell. Male and female controls did not show a significant difference in their fatigue score; similarly male and female cases did not differ significantly in their scoring of fatigue prior to the onset of their illness. Control students had a higher median fatigue score (median 3.5, i.e. more fatigued) than the score ascribed by cases prior to the onset of their symptoms (median 1.5) ($p<0.001$). Female students with IM (median score of 8) had significantly

higher fatigue scores than male cases at the time of their most severe symptoms (median 7) (p=0.011).

Students' assessment of their fatigue duration was available in 52/60 (87%) cases. The duration of their fatigue ranged from 3 days to 841* days (* still fatigued at last enquiry). The median duration of fatigue was 70 days, however, the duration of fatigue was longer in female students (median 125 days) than males (median 54 days, p=0.002) (Figure 13). When examined as a categorical variable fatigue at six months was significantly associated with female gender (p=0.005), but not with a stressful event in the six months before diagnosis (p=0.335) or with a diagnosis of mental illness in the preceding six months (p=0.114) (See Table 20). When fatigue is examined as a continuous variable (duration in days) students who reported a stressful event or mental illness in the 6 months prior to IM were more likely, although not significantly so, to experience a longer duration of fatigue (medians 94 days and 72 days respectively) (Table 21). In females this trend was more pronounced, with a median duration of 194 days in those reporting a prior stressful event (p=0.070), and a median duration of 263 days in those with a prior mental health complaint (p=0.247).

The initial score that students ascribed to the severity of their fatigue was positively correlated with the subsequent fatigue duration (p=0.021, see Figure 14) and the number of hours of timetabled university classes missed through illness (p<0.001, see Figure 15). The latter correlation remained significant when analysed by gender (males p=0.014, females p=0.016).

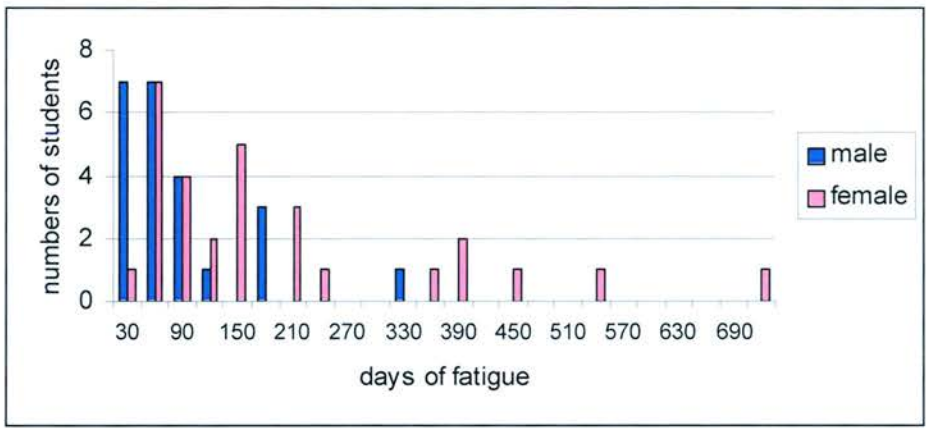


Figure 13 Duration of fatigue by gender

	Fatigue at 6 months n=12	No fatigue at 6 months n=41	p value
Male	1	22	p=0.005
Female	11	19	
Prior mental illness	5	7	p=0.114
Prior stressful event	6	14	p=0.335

Table 20 Fatigue at six months

n Number of students

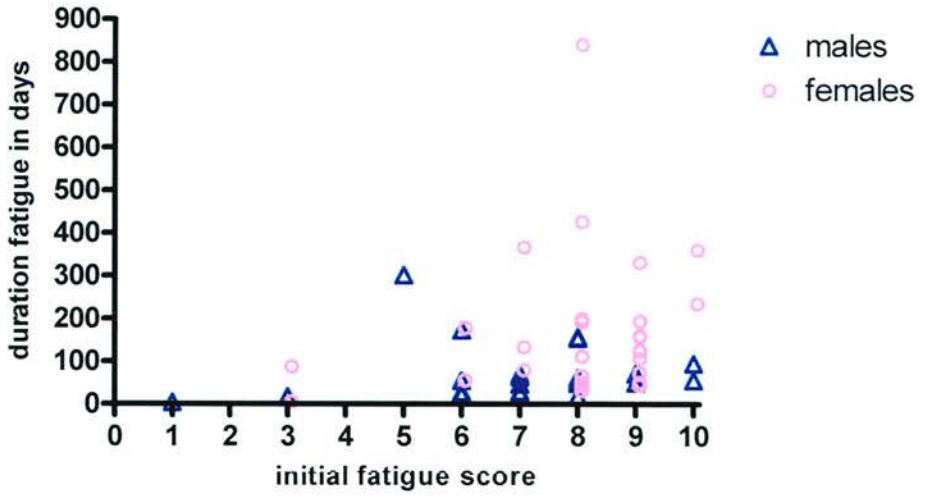


Figure 14 Initial fatigue score correlates with fatigue duration

p=0.021

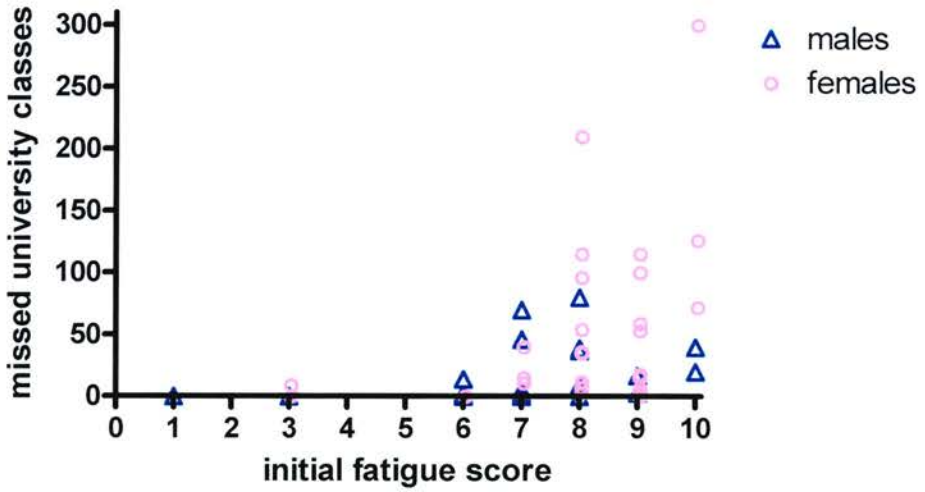


Figure 15 Initial fatigue score correlates with the number of hours of missed timetabled classes

p<0.001

Correlates of Fatigue duration

The presence of headache predicted a significantly longer duration of fatigue (median 94 days) than that observed in students without (median 48 days, p=0.009). A reported exercise tolerance of less than 100 meters at the time of the worst symptoms also predicted a longer duration of fatigue (medians 107 and 55 days, p=0.019) (see Table 21). Other features investigated did not attain statistical significance. When males and females were analysed together there was no significant correlation between the duration of fatigue and the viral load at diagnosis, the lymphocyte count at diagnosis, the reported weekly hours of exercise prior to IM and the number of days with symptoms prior to a diagnosis being made. There was a significant association between the duration of fatigue and the number of hours missed from university (p = 0.003), although this was more evident among females (p=0.011) than males (p=0.341) (Figure 16).

When males and females were analysed separately higher reported pre-morbid weekly hours of exercise in males correlated with a longer duration of fatigue (p=0.011, Figure 17).

	Number of students with variable	Median duration of fatigue if variable present	Median duration of fatigue if variable absent	p value
Fever	40/52	71 (5-841)	62 (3-367)	0.254
Sweating	38/52	71 (5-841)	62 (3-367)	0.392
Muscle aches	28/52	70 (17-525)	75 (3-841)	0.949
Headache	33/52	94 (17-841)	48 (3-427)	p=0.009
Anorexia	41/52	72 (5-841)	66 (3-367)	0.911
Nausea	7/52	51 (42-841)	70 (3-525)	0.872
Vomiting	7/52	160 (55-841)	66 (3-525)	0.099
Rash	7/52	80 (29-367)	70 (3-841)	0.758
Unable to follow usual diet	21/52	125 (5-427)	63 (3-841)	0.417
Unable to tolerate soft diet	15/52	94 (5-427)	69 (3-841)	0.960
Unable to leave home during worst symptoms	20/52	109 (21-841)	56 (3-525)	p=0.056
Walking distance <100m at worst	23/51	107 (21-841)	55 (3-367)	p=0.019
Onset of IM in vacation	12/52	130 (3-332)	68 (5-841)	0.837
Part-time term employment	10/45	75 (17-841)	70 (3-427)	0.702
Impaired concentration	40/52	71 (17-841)	59 (3-427)	0.318
Prior stressful event	19/52	94 (3-525)	66 (5-841)	0.556
Prior mental illness	11/52	72 (21-525)	69 (3-841)	0.453

Table 21 Clinical features of IM and duration of fatigue

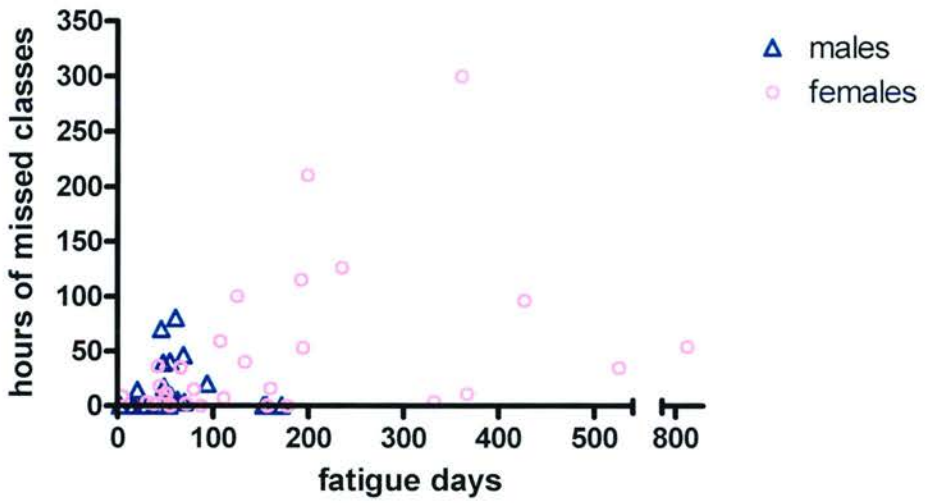


Figure 16 Hours of missed timetabled classes in males and females correlates with fatigue duration

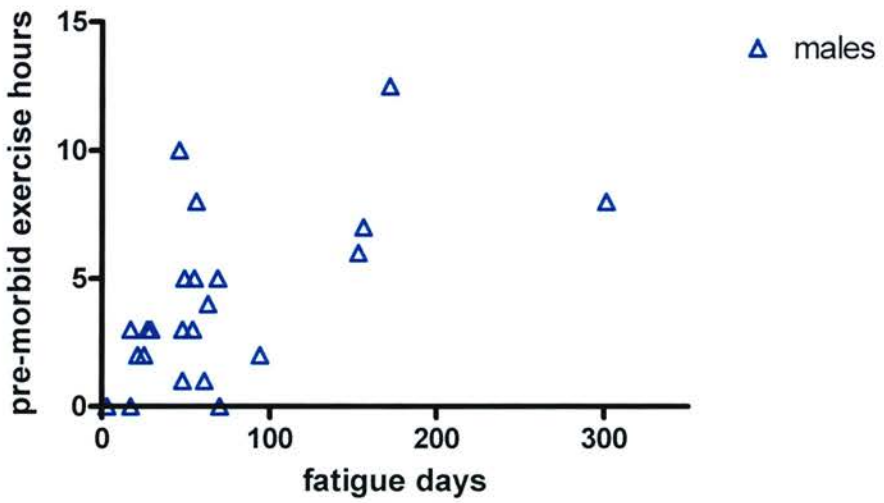


Figure 17 Weekly pre-morbid hours of exercise in males correlates with fatigue duration

7.9 *Medical, Lifestyle and Behavioural Characteristics*

The lifestyle and behavioural characteristics of cases and controls were compared to look for factors associated with IM.

7.9.1 Tonsillectomy

There was no significant difference in the proportion of cases and controls that had a prior history of tonsillectomy (p=0.666).

	Tonsillectomy	No history of tonsillectomy	Totals
Case	2	57	59
Control	3	50	53
Totals	5	107	112

Table 22 Tonsillectomy in IM cases and in controls

7.9.2 Psychological Illness

It has been suggested that psychological factors may be important in determining susceptibility or recovery in IM (Greenfield *et al*, 1959; Kasl *et al*, 1979). In view of this, cases and controls were asked if they had experienced a psychological or mental illness in the preceding six months. Thirteen (6M[1], 7F[2]) of 60 (22%) IM cases and 8 (3M, 5F) of 57 (14%) control students reported some form of psychiatric illness (p=0.282). Three students with IM and three control students had required treatment (depression 3, depression and overdose 1, depression and stress 1, stress 1).

7.9.3 Stressful Events

Students were asked if they had experienced any stressful events in the preceding six months, examples were: the death of or serious illness in a parent, partner, sibling or child; breaking off a marriage or steady relationship; a major financial crisis; financial pressure leading to part-time employment; or major problems with studies. Twenty five (14M, 11F) of the 60 of the IM cases (42%) had experienced a stressful event compared with 13 (4M, 9F) of 58 controls (22%) (p=0.025).

7.9.4 Paid Term-Time Employment

There was no statistically significant difference in the proportions of controls and cases

[1] M male

[2] F female

undertaking part-time paid employment during term-time. Thirty percent (17/57) of controls undertook paid work as did 26% (13/51) of the IM group (p=0.616). The IM cases worked a greater number of hours per week (median 15 hours) than the controls (median 11 hours) but this did not attain statistical significance (p=0.149).

7.9.5 Smoking and Alcohol Habits

There was no statistically significance difference in the proportion of cases or controls that stated they smoked or consumed alcohol.

	Case	Control	p value
Smoker	11	9	0.684
Non-smoker	49	49	
Consumes alcohol	59	53	0.111
Does not consume alcohol	1	5	
Number of alcoholic drinks per week	13.0	13.5	0.374

Table 23 Smoking and alcohol habits of cases and controls

7.9.6 Exercise

Six of 60 IM cases (10%) and 15/58 (26%) controls undertook no "vigorous" exercise (p=0.222). There was no significant difference in the number of hours of exercise per week reported by male (median 3 hours) and female controls (median 2 hours, p=0.274) or between male (median 3.5 hours) and female IM cases (median 4 hours, p=0.988) prior to the onset of IM. When the hours of exercise per week were compared in cases and controls, students that had developed IM reported a significantly greater number of exercise hours per week (before becoming unwell) than controls as discussed in 7.8.8.

7.9.7 Contact with Other Cases of IM

Students were asked if they knew anyone who had had glandular fever in the preceding three months. Twenty one of the 60 IM cases (35%) and 15 of 58 (26%) controls recalled such contact (p=0.281). In the IM group 5 of these contacts were partners, whereas none of the contacts recalled by the control group were partners.

7.9.8 Relationships

Cases and controls were asked about the total number of relationships or brief contacts, including kissing, they had had in the preceding three months. All cases (n=59, questionnaire incomplete for one case), but only 38/58 (66%) of the control group students had had a sexual relationship of any kind (including kissing) in the preceding three months (p<0.001). Six (10%) of the control group reported no previous sexual contact of any type including kissing at any time (p=0.013). Six cases (3 male and 3 female) and 2 controls

(both male) reported same sex relationships. Restricting the analysis to students reporting a relationship in the preceding 3 months revealed that, students with IM had, on average, a greater number of partners (mean 2.5) than the control group of students (mean 1.4), although the median number of contacts for both groups was one, (p=0.02 [Mann Whitney U]). The contact involved kissing only for 6 of 59 (10%) IM cases and 10/38 (26%) controls (p=0.036). In 20/59 (34%) students with IM and 4/38 (11%) controls the relationship had involved genital or orogenital contact, but not vaginal or anal intercourse (p=0.009). Amongst students that were sexually active, the proportions that had had penetrative sexual intercourse were similar: IM 33/59 (56%) and controls 24/38 (63%) (p=0.48).

7.9.9 Travel and Vaccination

The proportion of cases (30/60, 50%), and controls (30/58, 52%) that had travelled outside the UK in the last six months were similar, (p=0.851). There was no significant difference in the proportion of students that had received any form of vaccination in the preceding six months (cases 11/58, 19%, controls 18/58, 31%, p=0.133).

8 Analysis of Lymphocyte Subsets in Acute IM, Convalescence and IM-like Illness

In order to investigate the immune response to primary EBV infection the numbers and proportions of different lymphocyte subsets were studied using flow cytometry during acute and convalescent periods. These results were compared with data obtained from healthy control students and from students with "IM-like" symptoms. The latter were students thought to have symptoms consistent with acute IM by their GP but whose serology was negative for anti-VCA IgM and heterophile antibodies. Lymphocyte subset analysis was carried out on 73 occasions on 25 IM patients enrolled in the case control study, 12 controls, and 9 students with "IM-like" illness.

All of the acute IM cases had detectable anti-VCA IgM except one who had detectable heterophile antibody and had demonstrated anti-VCA IgG seroconversion. None of the controls or "IM-like" group had detectable anti-VCA IgM or detectable heterophile antibody. The results are summarised in Table 24. The results obtained from healthy controls in the current study are in line with other published data (see Appendix 12.4).

Time since diagnosis	Diagnosis	<15 days	15-30 days	1-2 months	2-4 months	≥ 5 months	"IM-like"	Control
Number of cases	n=14	n=6	n=6	n=5	n=8	n=13	n=9	n=12
Median count								
Total lymphocytes $x10^9\ l^{-1}$	7.00 p=<0.001	2.16 p=0.001	1.67 p=0.053	1.61 p=0.430	1.87 p=0.021	1.56 p=0.058	1.54 p=0.789	1.39
$CD3^+\ x10^9\ l^{-1}$ (T-cells)	5.15 p=<0.001	1.46 p=0.035	1.0 p=0.963	1.12 p=0.493	1.19 p=0.177	1.26 p=0.077	0.79 p=0.303	1.02
$CD8^+\ x10^9\ l^{-1}$ (CD8+ T-cells)	4.53 p=<0.001	0.57 p=0.028	0.4 p=0.888	0.39 p=0.874	0.52 p=0.232	0.48 p=0.165	0.36 p=0.374	0.41
$CD4^+\ x10^9\ l^{-1}$ (T-helper cells)	0.92 (n=13) p=0.370	0.46 p=0.101	0.41 p=0.068	0.63 p=0.370	0.60 p=0.563	0.70 p=0.849	0.54 p=0.110	0.75
$\gamma\delta\ x10^9\ l^{-1}$ ($\gamma\delta$ lymphocytes)	0.46 (n=11) p<0.001	0.32 p=0.001	0.20 p=0.006	0.15 p=0.044	0.32 p<0.001	0.15 p=0.036	0.11 p=0.526	0.08
$CD56^+CD3^-$ $x10^9\ l^{-1}$ (NK cells)	1.00 (n=13) p=<0.001	0.50 p=0.008	0.33 p=0.013	0.22 p=0.126	0.31 p=0.028	0.22 p=0.087	0.21 p=0.145	0.14
$CD19^+x10^9\ l^{-1}$ (B cells)	0.26 p=0.857	0.14 p=0.325	0.17 p=0.147	0.13 p=0.031	0.27 p=0.729	0.21 p=0.242	0.26 p=0.749	0.27
CD4%/CD8%	0.18 p=<0.001	0.70 p=0.006	1.27 p=0.211	1.23 p=0.726	1.19 p=0.129	1.31 p=0.311	1.41 p=0.643	1.57

Table 24 Lymphocyte subset analysis in acute IM, convalescents, controls and IM-like illness

8.1.1 Total Lymphocyte Counts

The median total lymphocyte counts of the acute IM group (7.0 $x10^9l^{-1}$) was significantly greater than that of the control group (1.39 $x10^9l^{-1}$, p<0.001), (see Table 24 and Figure 18). The median lymphocyte count of the heterophile negative, "IM-like" group (1.54 $x10^9l^{-1}$) was significantly less than that of the acute IM group (p<0.001) but was not significantly different from the control group (p=0.789). The median lymphocyte counts of convalescing IM cases continued to exceed that of the control group at all time points (see Figure 18). This difference was statistically significant in the first 15 days (2.16 $x10^9l^{-1}$, p=0.001), 15-30 days (1.67 $x10^9l^{-1}$ p=0.05) and also in the group collected between 2 and 4 months after

diagnosis (1.87 $x10^9l^{-1}$, p=0.021).

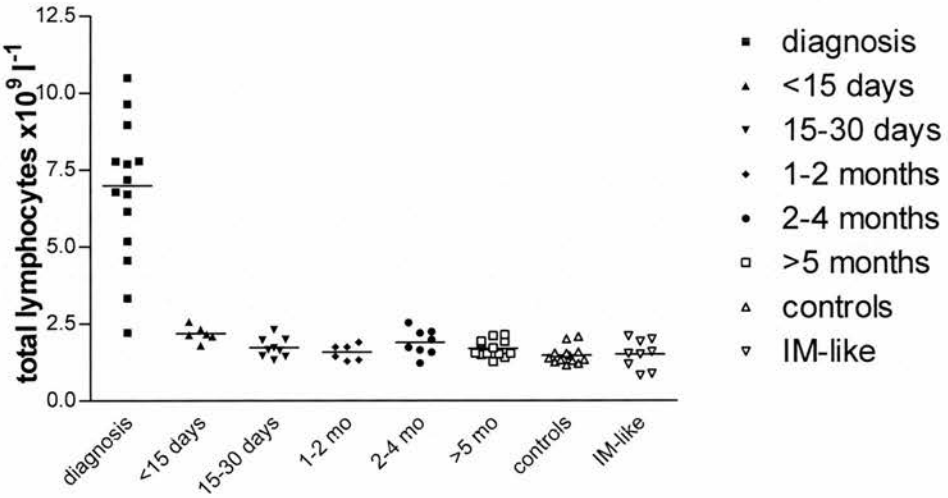


Figure 18 Total lymphocyte numbers in IM, convalescents, controls and IM-like illness

8.1.2 T-lymphocytes ($CD3^+$)

T-lymphocyte numbers in the acute IM group (median 5.15 $x10^9l^{-1}$) significantly exceeded those of the control group (1.02 $x10^9l^{-1}$, p<0.001) and those of the "IM-like" group (0.79 $x10^9l^{-1}$, p<0.001) (see Table 24 and Figure 19). The median $CD3^+$ T-cell count of the "IM-like" group did not differ significantly from that of the control group. Samples from six students convalescing from IM who were seen within two weeks of diagnosis, had T-cell numbers greater than those of the control group (1.46 $x10^9l^{-1}$, p=0.035). After this period $CD3^+$ T-cell numbers of the convalescent students did not differ significantly from those of the controls.

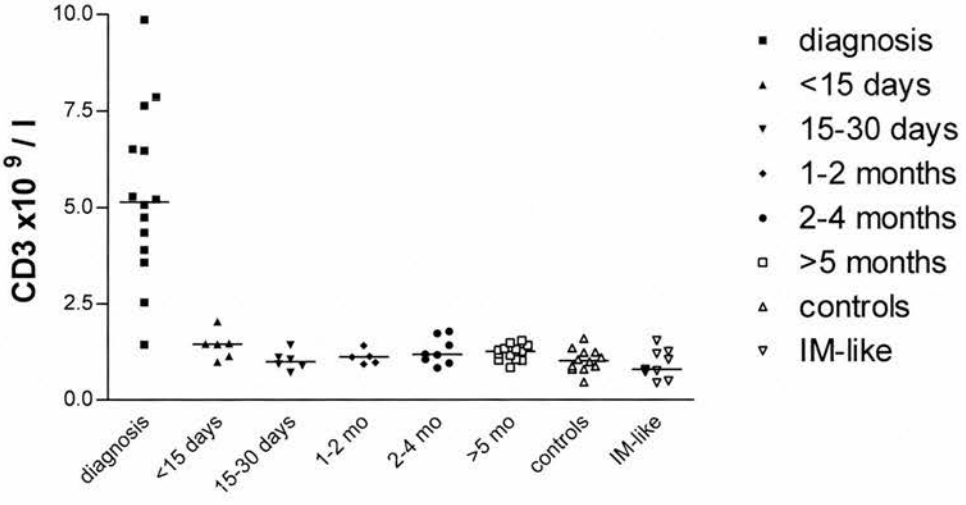


Figure 19 T-lymphocyte numbers in IM, convalescents, controls and IM-like illness

8.1.3 CD8+ T-cells

$CD8^+$ T-cell numbers at diagnosis (median 4.53 $x10^9l^{-1}$) were significantly greater than those of the control group (0.41 $x10^9l^{-1}$, $p<0.001$) and those of the "IM-like" group (0.36 $x10^9l^{-1}$, $p<0.001$) (see Table 24, Figure 20 and Figure 21). Only one IM sample fell within the range of the control group (IM range 0.65-9.08 $x10^9l^{-1}$, control range 0.21 – 0.95 $x10^9l^{-1}$). The median $CD8^+$ count of the "IM-like" group (0.36 $x10^9l^{-1}$) did not differ significantly from that of the control group. For a group of six cases, where samples were collected within 15 days of diagnosis, the median $CD8^+$ T-cell count (0.57 x 10^9 l^{-1} ^{1}) was significantly greater than that of the control group, ($p=0.028$). The convalescent data demonstrate that $CD8^+$ T-cell numbers decrease rapidly with results two or more weeks after diagnosis being close to control values.

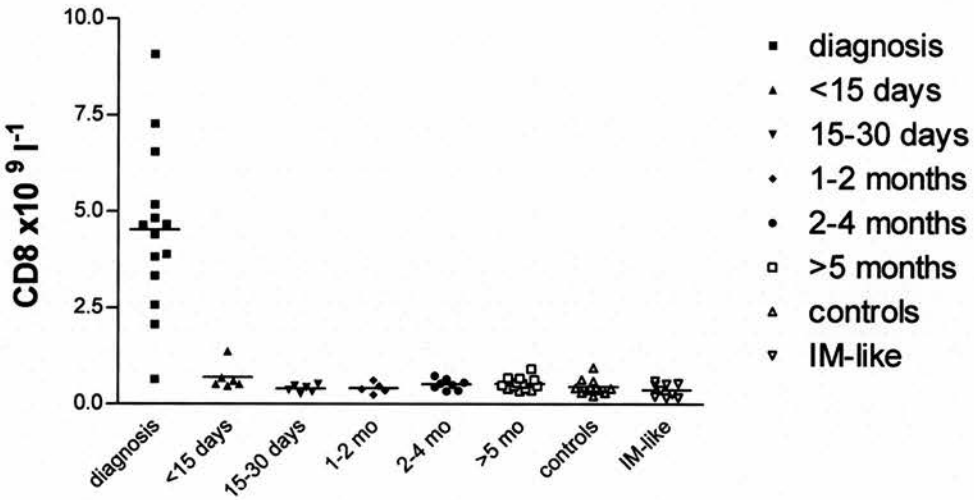
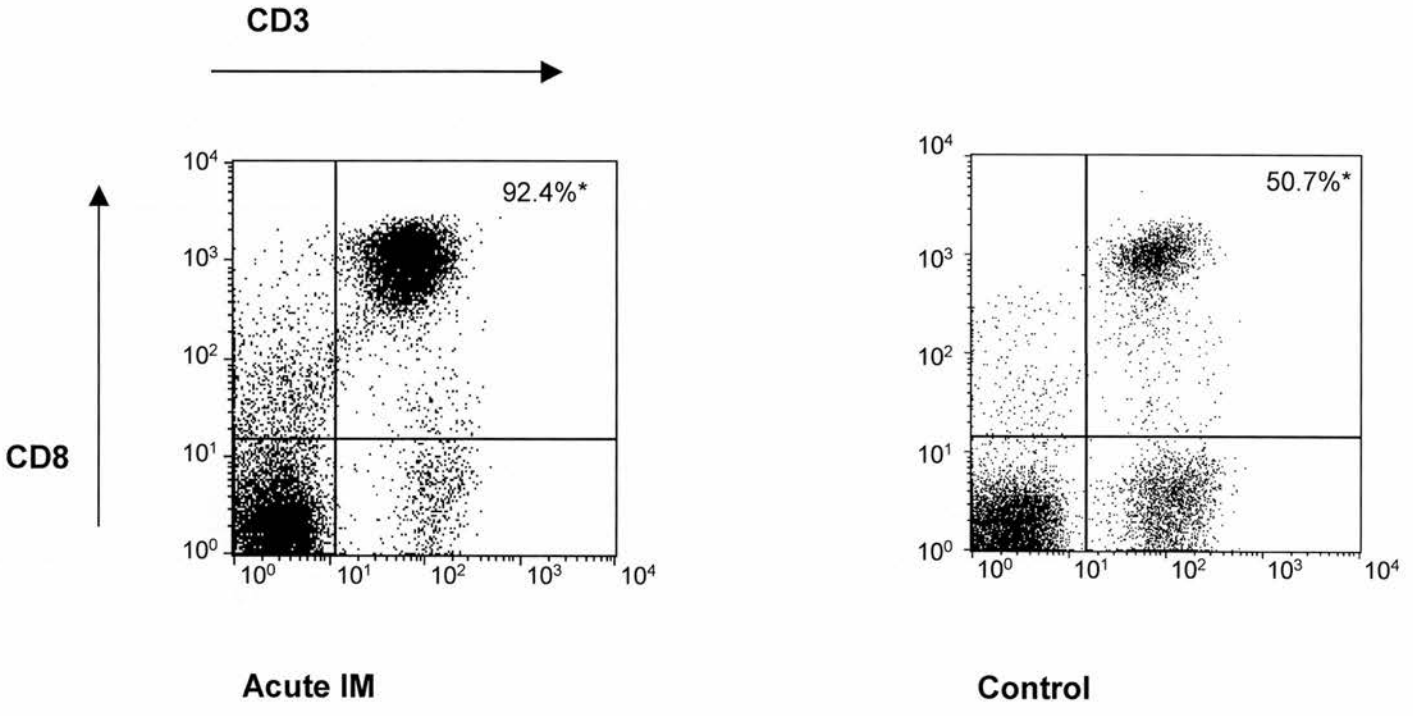


Figure 20 CD8^{+} T-cell numbers in IM, convalescents, controls and IM-like illness

Figure 21
$CD8^+$ T-cells in acute IM and a healthy control



* As a percentage of CD3

8.1.4 T-helper cells ($CD4^+$ $CD3^+$)

$CD4^+$ T-cell numbers of the acute IM group (median 0.92 $x10^9l^{-1}$) did not differ from those of the control group (median 0.75 $x10^9l^{-1}$) (see Table 24, Figure 22 and Figure 23). Figure 23 demonstrates the importance of measuring actual lymphocyte subset numbers in acute IM, because the different lymphocyte populations are not altered equally. Thus the proportion of T-cells that are $CD4^+$ falls whilst the actual numbers of these cells remains almost constant. The median $CD4^+$ counts of the convalescent IM groups and the "IM-like" (0.54 $x10^9l^{-1}$) were all less than the control group, but this did not attain significance for any of the groups, although the median $CD4^+$ count of students seen between 15 and 30 days after diagnosis (0.41 $x10^9l^{-1}$) bordered on being significantly lower than that of the control group (p=0.068).

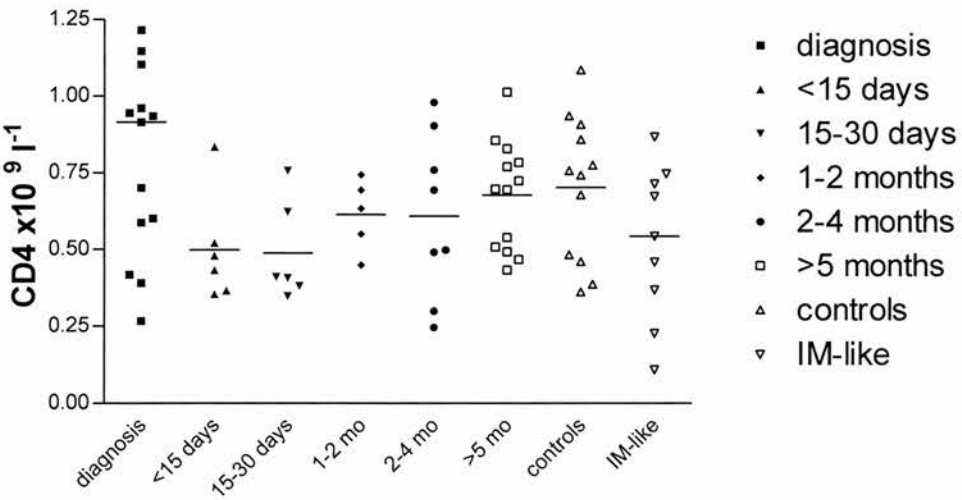
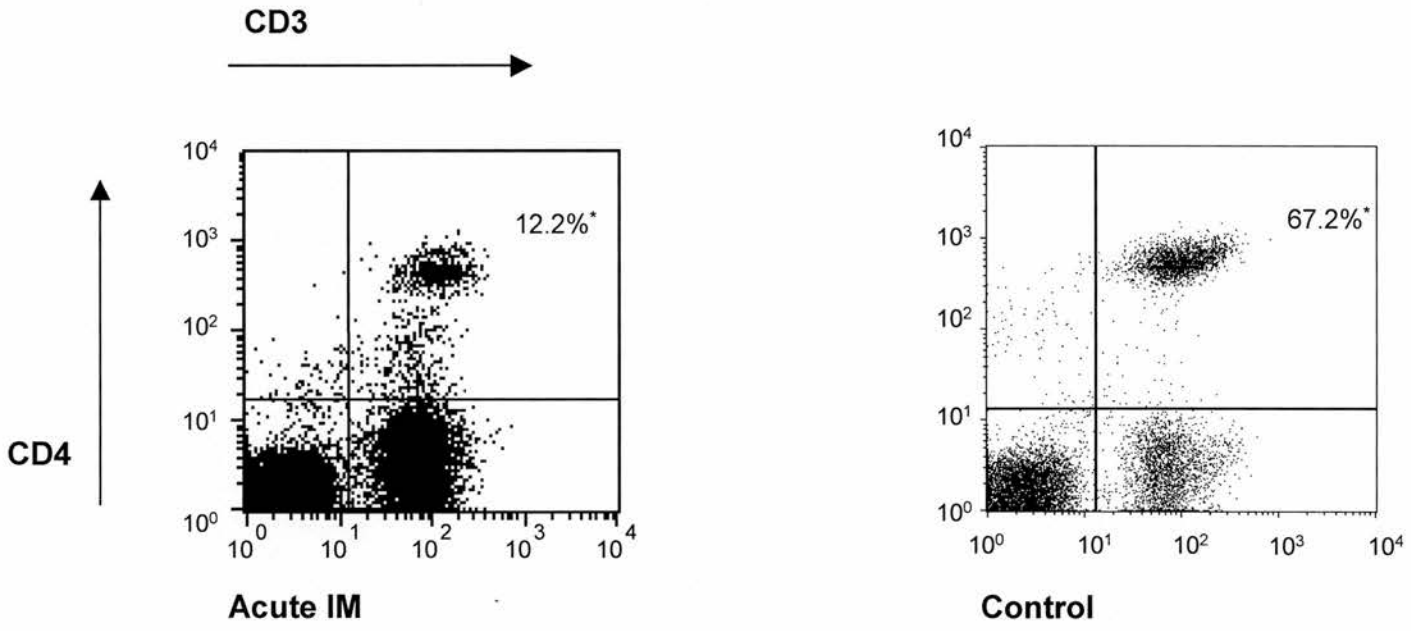


Figure 22 $CD4^+$ T-cell numbers in IM, convalescents, controls and IM-like illness

Figure 23
$CD4^+$ T-cells in acute IM and a healthy control



* As a percentage of CD3

8.1.5 Gamma-delta T-cells

Gamma-delta T-cell numbers at diagnosis (median 0.46 $x10^9l^{-1}$) were significantly greater than in the control group (0.08 $x10^9l^{-1}$, $p<0.001$) and the "IM-like" group (0.11 $x10^9l^{-1}$, $p=0.004$) (Table 24). The γδ T-cell numbers of the "IM-like" group and the control group did not differ significantly. Gamma-delta T-cell numbers of the IM group remained significantly greater than those in the control group at all time points when the convalescent group was divided up according to the time elapsed since diagnosis (see Table 24 and Figure 24). Samples from thirteen students, taken at least 5 months after the diagnosis of IM (median 0.15 $x10^9l^{-1}$), still showed a significant elevation when compared with the control group ($p=0.036$).

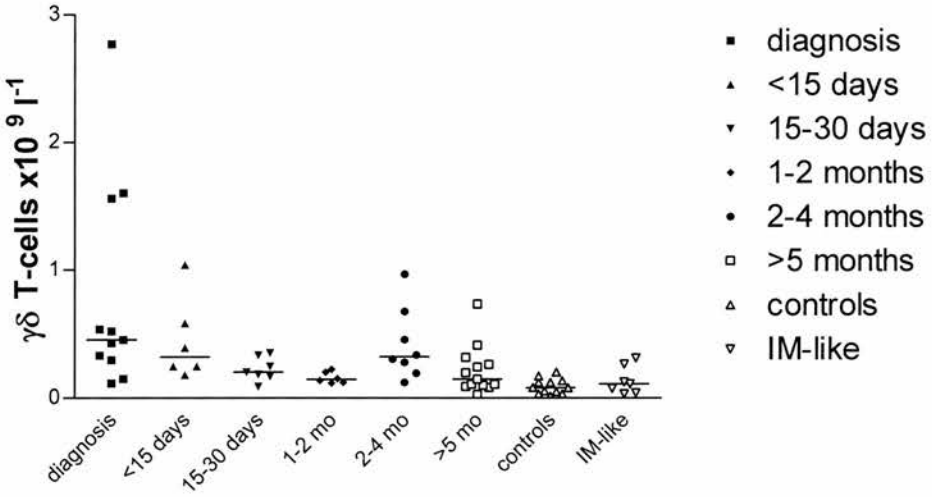
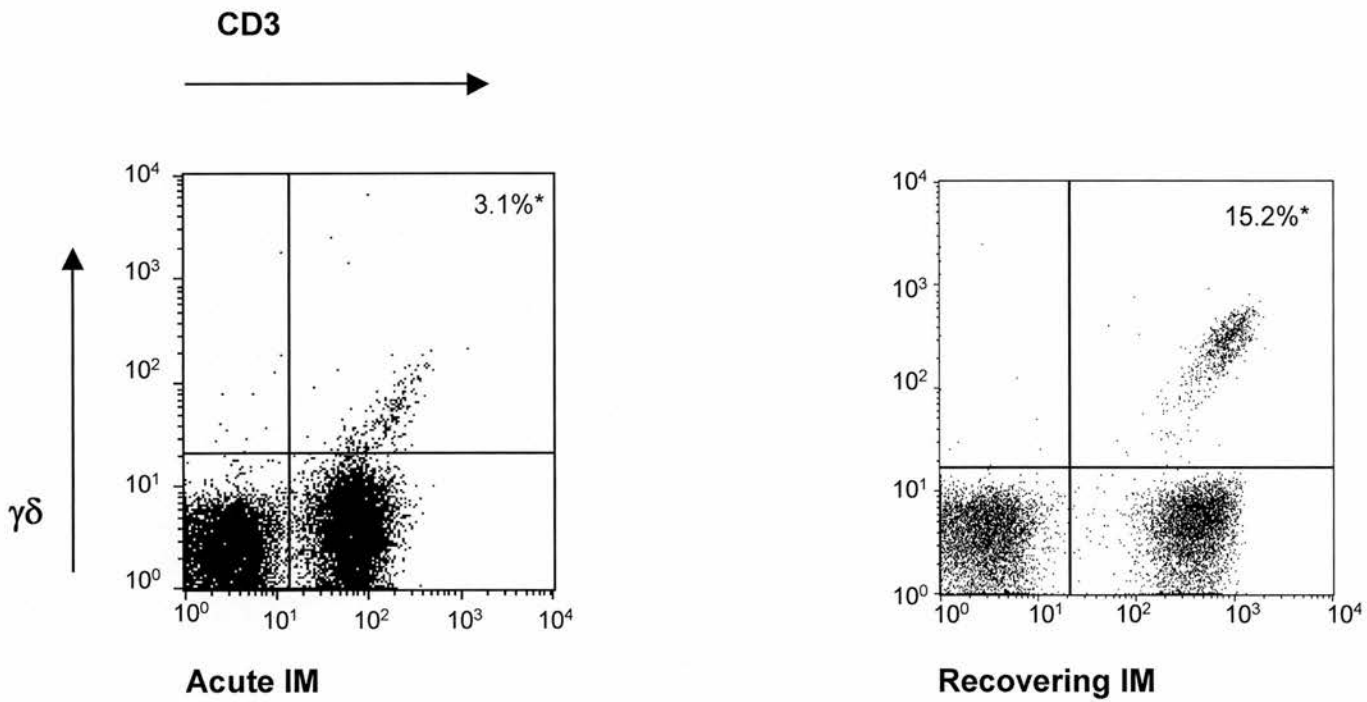


Figure 24 Gamma-delta T-cell numbers in IM, convalescents, controls and IM-like illness

Figure 25
Gamma-delta T-cells in acute IM and in the convalescent period



*As a percentage of CD3

8.1.6 Natural Killer cells ($CD56^+CD3^-$)

NK cells were identified as staining positively for CD56 but negatively for CD3 (see Figure 27). The results (Table 24 and Figure 26) exclude the small population of cells positive for both markers. NK cell numbers at diagnosis in IM (median 1.0 $x10^9l^{-1}$) were greater than those of the control group (0.14 $x10^9l^{-1}$, $p<0.001$) and the "IM-like" group (0.21 $x10^9l^{-1}$, $p<0.001$). NK cell numbers of the "IM-like" group did not differ significantly from those of the control group. NK cell numbers exceeded those of the control group at all time points after diagnosis, and this difference was significant for samples collected from students within 15 days of diagnosis (0.5 $x10^9l^{-1}$, $p=0.008$), 15-30 days (0.33 $x10^9l^{-1}$, $p=0.013$) and 2-4 months after diagnosis (0.31 $x10^9l^{-1}$, $p=0.028$).

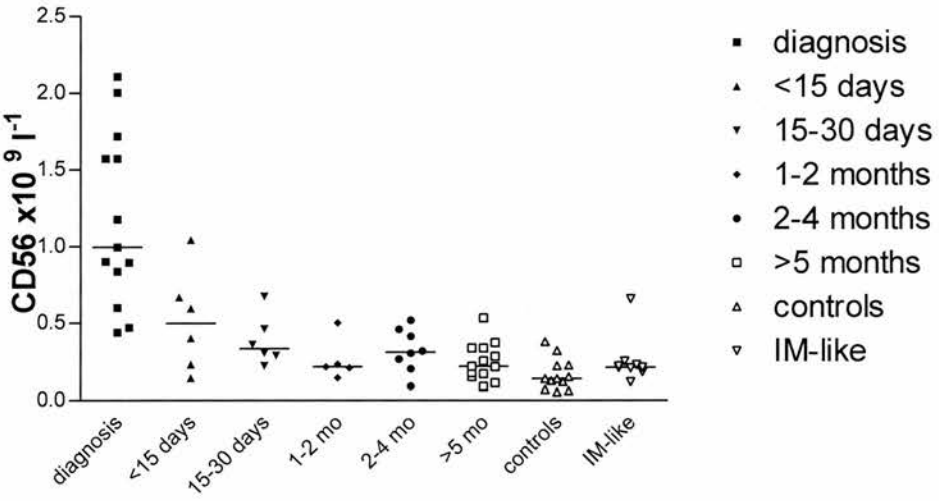
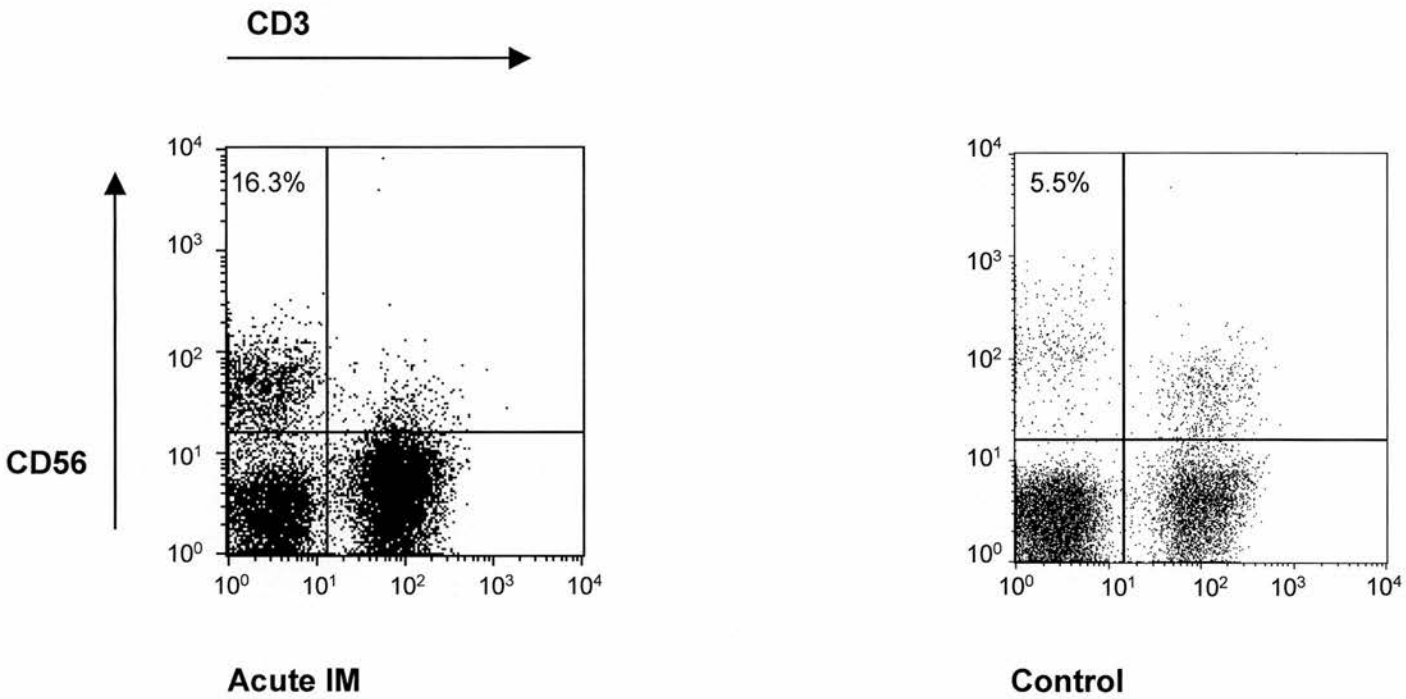


Figure 26 $CD56^+$ NK cell numbers in IM, convalescents, controls and IM-like illness

Figure 27
CD56$^+$ NK cells in acute IM and a healthy control



8.1.7 B-Cells (CD19+)

B-cell numbers at diagnosis (0.26 $x10^9l^{-1}$) and in the "IM-like" group (0.26 $x10^9l^{-1}$) did not differ significantly from the control group (0.27 $x10^9l^{-1}$). B-cell numbers were reduced in the convalescent period up to 2 months after IM when compared with controls (Table 24 and Figure 28). This difference was significant for a group of 5 samples collected at 1-2 months after diagnosis (median 0.13 $x10^9l^{-1}$. p=0.013).

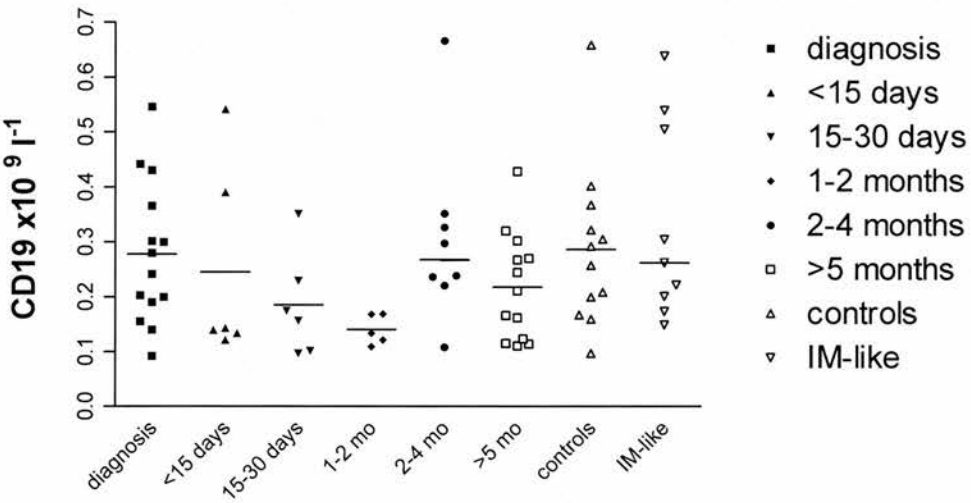


Figure 28 B-cell numbers in IM, convalescents, controls and IM-like illness

8.1.8 CD4/CD8 Ratio

At the time of IM diagnosis the number of $CD8^+$ T-cells was generally greatly increased whilst the number of $CD4^+$ T-cells was maintained at normal levels, which resulted in inversion of the normal ratio of $CD4^+$ to $CD8^+$ T-lymphocytes. The ratio of $CD4^+$ to $CD8^+$ T-cells in controls gave a ratio of 1.57, whilst at IM diagnosis the ratio was 0.18 ($p<0.001$), (see Table 24 and Figure 29). The abnormal ratio persisted for 2 weeks after diagnosis. Controls (1.57) and the "IM-like" group (1.41) did not differ significantly in their CD4/CD8 ratios.

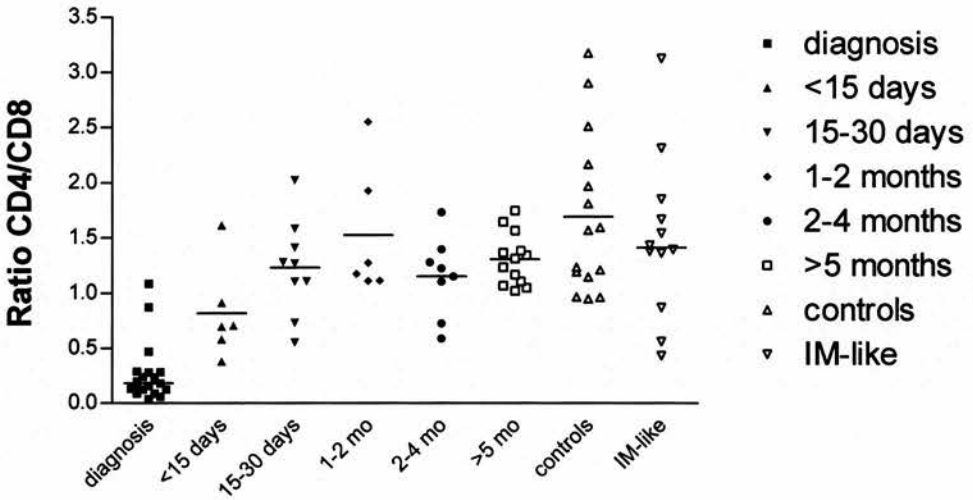


Figure 29 CD4/CD8 Ratio in IM, convalescents, controls and IM-like illness

9 Discussion

After Epstein-Barr virus was recognised as the cause of infectious mononucleosis in 1968 (Henle *et al*, 1968) several studies were undertaken to investigate the epidemiology of EBV infection and IM (Anon., 1971; Biggar *et al*, 1978; Dan & Chang, 1990; de-Thé *et al*, 1978; Hallee *et al*, 1974; Kafuko *et al*, 1972; Lehane, 1970; Niederman *et al*, 1970; Sawyer *et al*, 1971). These showed that the annual rate of seroconversion in seronegative populations ranged between 2% (troops in Vietnam) and 27% (peace corps volunteers). The proportion experiencing symptomatic seroconversion also ranged widely between none (students attending the Chinese University of Hong Kong and a study including peace corps volunteers) and 74% (students at Yale University). The clinical features of IM were studied at this time but these observations were based on distinct populations of hospitalised college students or military personnel that were comprised almost entirely of males (Dalrymple, 1964; Finkel *et al*, 1964; Hallee *et al*, 1974; Hoagland, 1960). Additionally some clinical studies, notably those of Hoagland, predated the discovery of EBV and its association with IM, and confirmation of IM diagnosis included strict haematological criteria of 50% lymphocytosis with atypical lymphocytosis of 10%, as well as the presence of heterophile antibody (Hoagland, 1960; McCarthy & Hoagland, 1964). Later commentators have found that these criteria only have a sensitivity of between 39 and 43% for a single blood count (Fleisher *et al*, 1983; McNeely, 1991). Thus some cases may have been excluded from earlier descriptions, particularly those with mild or atypical symptoms, although the numbers potentially excluded are probably not as high as 60% because the patients were hospitalised and serial blood counts were performed. In recent years three clinical studies discussing the clinical features of IM have been published, one conducted by The Israeli Defence Force Medical Corps, another based in a large health maintenance organisation in Seattle, US, which included 2 hospitals and 23 outpatient clinics, and most recently a small study of 25 cases also from the US (Balfour *et al*, 2005; Grotto *et al*, 2003; Rea *et al*, 2001). Of note, two thirds of the cases in the Seattle based study were students perhaps emphasising the importance of this group in the disease epidemiology overall.

The recognition that EBV is associated with a number of malignancies particularly nasopharyngeal carcinoma, Burkitt's lymphoma, post transplant lymphoproliferative disease and Hodgkin's Disease, and that in addition IM is a recognised risk factor for the latter condition (Hjalgrim *et al*, 2003; Robertson, 2005) has reawakened interest in immunisation to prevent IM and malignancy (Balfour, 2007; Moss *et al*, 1998; Sokal *et al*, 2007; Thompson & Kurzrock, 2004). The present study was undertaken to investigate the current

epidemiology of EBV, the risk factors for symptomatic seroconversion and the clinical and social impact of symptomatic infection.

Baseline Serological Characteristics

A total of 2006 first year university students were recruited at the start of the academic years 1999 and 2000. These students underwent serological testing for EBV and answered an epidemiological questionnaire. After 3 years of study all students were invited to attend for follow-up serological testing and to complete a further questionnaire. At initial recruitment 75% of the cohort, which had a median age of 18.8 years, were EBV seropositive. Male students were more likely to be seronegative (31%) than female students (21%). A cross-sectional survey of EBV serostatus in England and Wales reported a similar findings, with males in the 10-14 and 15-19 age groups having a lower seroprevalence than females, however, this differential was not found in the 20-24 group (Morris *et al*, 2002). The reason for the delayed acquisition in males is not known but would appear not to be explained by earlier sexual activity in females. *The National Survey of Sexual Attitudes and Lifestyles* found an earlier age of "any type of experience of a sexual kind – for example, kissing, cuddling, petting" in males (median 13 years in males and 14 years in females in the cohort aged 16-24) and a higher proportion of males experiencing first sexual intercourse before 16 years (28% for males and 19% for females in the cohort aged 16-24), however, the median age of first sexual intercourse was 17 years for both genders in the same cohort (Wellings *et al*, 1994). Several potential explanations exist for this later acquisition of antibody in males; females are known to select male partners older than themselves (Johnson *et al*, 1990; Wadsworth *et al*, 1993), who as a consequence of being older are more likely to be seropositive; alternatively the difference may reflect other social or behavioural differences in male and female adolescents; or, if vaginal sexual intercourse is a significant route of transmission then it is also possible that women may be more likely to acquire EBV sexually than men (as has been observed in Western studies of HIV seroconversion in couples with discordant HIV status (Mastro & Kitayaporn, 1998; Nicolosi *et al*, 1994), and also in seroprevalence surveys of HSV-2 (Gibson *et al*, 1990; Morris *et al*, 2002; Pebody *et al*, 2004; Slomka *et al*, 2002; Vyse *et al*, 2000)).

The baseline lifestyle questionnaire in the current LCS did show that having acquired EBV at the time of university entrance was strongly associated with prior sexual intercourse, however, there was no difference in the proportion of male and female students that had been sexually active prior to university. The likelihood of having acquired EBV increased with increasing numbers of previous sexual relationships; from 63% EBV seroprevalence for students who had never been sexually active to 91% seroprevalence in students reporting 5

or more previous sexual partners. Those students reporting prior IM were more likely to have had sexual intercourse prior to university, than students who were seropositive without experiencing IM, or students who were seronegative.

EBV DNA has been detected in breast milk (Junker *et al*, 1991), however, the transplacental transmission of maternal antibodies to EBV would appear to protect infants against early infection as evidenced by the abrupt onset of seroconversion after 8 months of age (Chan *et al*, 2001), perhaps explaining the absence of an association between EBV and breast feeding. In the current seroepidemiological study there was no association with breast feeding, however, the proportion of students reporting that they were breast fed seems improbably high at 92%. A survey conducted in 2005 found that only 7% of UK infants were exclusively breast fed until 4 months of age, whilst comparable rates in Norway are 64% (Hoddinott *et al*, 2008).

Those who had resided in a tropical country were significantly more likely to have acquired EBV (81%) compared with those who had not (73%). The association was stronger for students born in a tropical country, 84% of whom were EBV seropositive. These finding are in keeping with epidemiological studies conducted in Africa (Biggar *et al*, 1981; Kafuko *et al*, 1972), China (Wang & Evans, 1986), and India (Venkitaraman *et al*, 1985), showing a high sero-prevalence of EBV in early childhood. No significant association was found between ethnicity and EBV seroprevalence in our study although it should be noted that the cohort was 94% Caucasian, resulting in small numbers for the comparison.

As in a prior epidemiological investigation (Lai *et al*, 1975) those with siblings were significantly more likely to be seropositive (75%) than those without (66%), although not all workers have found this association (Dan & Chang, 1990).

No association was found between EBV seropositivity and sharing a bed room or household crowding (as measured by the average number of rooms per person in the household prior to university). Previous seroepidemiological surveys have yielded mixed results. Crowcroft *et al* (1998) found that sharing a bed room was associated with EBV seropositivity after multivariate analysis, but that the effect was most marked in families of lower socio-economic status. Household size was not significantly associated with EBV seropositivity after multivariate analysis. Another study found no consistent relationship between EBV seropositivity, household size and other measures of crowding (Sumaya *et al*, 1975). Two further prospective studies of IM found no association with household crowding (Dan & Chang, 1990; Hallee *et al*, 1974).

EBV Seroconversion at University

Eight hundred and forty two students returned for follow-up at the end of the study including

241 (47%) of the initially seronegative students. Of the seronegative students that returned 110 (46%) had seroconverted, and 27 (25%) of these had contracted IM. This is at the lower end of earlier higher education institution based studies in the UK and US where the proportion of symptomatic EBV seroconversion ranged between 28 and 74% (Anon., 1971; Hallee *et al*, 1974; Niederman *et al*, 1970; Sawyer *et al*, 1971) (see Table 2). In a study based in the University of Hong Kong all EBV seroconversions were asymptomatic, and studies in American military personnel found either no cases of IM or a low rate based on hospitalisation of cases (Dan & Chang, 1990; Lehane, 1970; Niederman *et al*, 1970). In our cohort there was an annual EBV seroconversion rate of 15% and an annual IM rate of 4% amongst the seronegative students. These rates are in the range of those noted in previous studies (see Table 2) where annual seroconversion rates in institutions ranged between 7% and 27%, and annual IM rates ranged between zero in Hong Kong and 13% in students at Yale University (Anon., 1971; Dan & Chang, 1990; Hallee *et al*, 1974; Lehane, 1970; Niederman *et al*, 1970; Sawyer *et al*, 1971). As in the baseline observations of the LCS EBV seroconversion at university was significantly associated with an increased number of sexual partners.

In the case control study, cases, before they became unwell, reported a significantly greater number of hours undertaking vigorous exercise every week than control students. Whilst this may reflect recall bias by the IM cases there was no difference between cases and controls in the reported hours spent on social activities or studying. Thus perhaps exercise may predispose to symptomatic, rather than silent, EBV seroconversion. A parallel can be drawn with polio where exercise often appeared to precipitate a severe or fatal paralysis, or, paralysis in which the muscles exercised were more severely affected (Russell, 1949). Polio that occurred following muscle injury, exercise, or intramuscular injections, was known as "provocation poliomyelitis", and more recently this form of the disease was studied in a transgenic mouse model. It is thought that CD155, a cellular receptor of unknown function, to which Polio virus binds, is upregulated in nerve endings following minor trauma (Gromeier & Wimmer, 1999).

Presenting Symptoms in IM

The commonest symptoms which caused the students to attend their GP were sore throat, fatigue and symptoms related to "swollen glands". On direct questioning only two of 60 students did not experience a sore throat, although they were aware of swelling of their cervical lymph nodes. Hoagland in his review of 200 IM cases found lymph node enlargement and fatigue in all IM cases but reported that 12% of patients had a "typhoidal" presentation without pharyngitis or jaundice (Hoagland, 1960). Hoagland also considered

that certain symptoms including cough, sputum, joint pains, painful or extremely tender lymph nodes, and severe abdominal pain mitigated "against the diagnosis of uncomplicated mononucleosis". It should be noted that specific EBV serology was not available to Hoagland and that the cases described may have been restricted as discussed previously. More recent studies using specific anti-VCA IgM as the diagnostic criterion have reported cough in 22% of cases, painful lymphadenopathy in 57%, "nausea/vomiting" in 41%, headache in 50-83%, and "myalgia / arthralgia" in 32% (Grotto *et al*, 2003; Rea *et al*, 2001). The current study found few students complained of fever (13%), and that some had presentations which included atypical symptoms including gastrointestinal symptoms 8% (predominantly vomiting), headache 10%, fainting 10% or cough 7%. Acute symptoms lasted a median of 7 days in both genders, and were followed by a fatigue state lasting a median of 70 days, which lasted more than twice as long and was subjectively more severe in female students (see below).

Examination Findings

The majority of cases (88%) had palpable cervical lymphadenopathy, significantly more than that detected in control students (23%). Hoagland (1960) found lymph node enlargement in all his cases, however, as noted earlier his cases were based on different diagnostic criteria and the patient population comprised young male soldiers. More recent reports noted cervical lymphadenopathy in 77-89% of cases (Grotto *et al*, 2003; Rea *et al*, 2001). In the present study splenomegaly or hepatosplenomegaly was only found in cases and not in controls. Of IM cases reviewed within two weeks of diagnosis 35% had splenomegaly or hepatosplenomegaly, which is in line with the findings of Finkel *et al* (1964) who found splenomegaly in 38% of 235 patients. In the current study the proportion of cases (12%) and controls (14%) that had isolated hepatomegaly was similar. The presence of a palpable liver or spleen in cases was significantly associated with elevation of ALT, whilst a palpable liver in controls was not associated with derangement of liver function tests.

Concurrent prescription of ampicillin or amoxicillin is well known to cause rash in IM (Johannsen *et al*, 2005), however, of the eight students (13%) that experienced a skin rash as part of the illness only two were taking antibiotics, demonstrating that rash is regularly a feature of the illness itself. This study found rash in a greater proportion of cases than the 3% noted by Hoagland (McCarthy & Hoagland, 1964), although other workers have also recorded rash in 15-17% of IM cases (Balfour *et al*, 2005; Grotto *et al*, 2003; Pullen *et al*, 1967; Rea *et al*, 2001). Five percent of students suffered very severe acute symptoms requiring hospital admission or attendance.

Laboratory Parameters

The diagnosis of acute IM was confirmed with anti-VCA IgM and or a positive heterophile antibody plus demonstrated anti-VCA IgG seroconversion. The rapid slide test for heterophile antibody used (Microgen i.m. absorption kit) had excellent sensitivity in this population with only one test (2%) negative at the time of diagnosis, and this had become positive on repeat testing three weeks later. Anti-VCA IgM was negative at the time of the initial sample on four occasions (8%) and became positive on later repeat testing in two, and remained negative in two. The specificity, and, positive and negative predictive values of the heterophile antibody test and anti-VCA IgM were not assessed in this study. False positive and negative heterophile results are well recognised; positive results may occur in other infections, including HIV (of concern because HIV seroconversion illness may appear clinically similar to IM), inflammatory conditions and also as an incidental finding (Hendry & Longmore, 1982; Horwitz *et al*, 1979; Reed, 1974; Ridker *et al*, 1990; Seitanidis, 1969; Vidrih *et al*, 2001; Wynne Jones *et al*, 1994). False negative results are particularly associated with young children (Sumaya & Ench, 1985). Whilst the sensitivity of different kits varies it is generally accepted that the use of horse RBC is the most sensitive means of detecting heterophile antibody (Beer, 1936; Evans *et al*, 1975; Lee *et al*, 1968b; Linderholm *et al*, 1994). In the current study the rapid slide test for heterophile antibody remained positive for longer than anti-VCA IgM, with 22% of IM cases positive after 5 months, as opposed to 13% for anti-VCA IgM. This is in keeping with earlier work using a plate method to detect horse RBC heterophile agglutinins which showed 73% were still positive at one year whilst only 25% of the anti-VCA IgM remained positive (Evans *et al*, 1975). Also in line with our findings a more recent study found that only 13% of IM cases had detectable anti-VCA IgM at six months (Rea *et al*, 2001). Anti-VCA IgG was present in all but one student (98%) at diagnosis and this student had developed anti-VCA IgG on repeat testing 8 days after initial presentation.

Acute IM is typically associated with a marked lymphocytosis and the presence of atypical lymphocytes on the blood film. In the current study 75% of cases had a lymphocytosis (defined as $> 4x10^9\ l^{-1}$) at the time of diagnosis, whilst 23% had lymphocyte counts within in the normal range, and one case had a count below the normal range. None of the healthy control students had a lymphocytosis, in fact almost half of them were classified as lymphopenic ($<1.5\ x10^9\ l^{-1}$) according to the laboratory's "normal range". In IM cases the lymphocyte count remained significantly greater than that of the control students for up to thirty days following diagnosis.

Twenty six percent of cases and 7% of controls had neutrophil counts less than $2x10^9\ l^{-1}$,

which is the lower limit of the normal range. Only IM cases had neutropenia of less than $1x10^9$ l^{-1} which occurred in four students (9%). When compared with controls, cases had significant reductions in their median neutrophil count for up to 2 months after diagnosis. Reduced neutrophil counts were previously reported by Carter who found 4% of 172 acute IM cases had counts of less than $1x10^9$ l^{-1} (Carter, 1966a). Increased cytokine-induced apoptosis, direct cell invasion and anti-neutrophil antibodies have been suggested as causes for the reduced neutrophil count in acute IM (McAulay *et al*, 2007; Savard & Gosselin, 2006; Schooley *et al*, 1984).

Thrombocytopenia is also a recognised complication of acute IM and although platelet counts below the normal range were noted in 21% at diagnosis, none were less than $100x10^9$ l^{-1} and all were asymptomatic. All but one of 43 retested within a month had a platelet count above the lower limit of the normal range. Asymptomatic thrombocytopenia has previously been reported in 24-50% of cases (Cantow & Kostinas, 1966; Carter, 1965) but this has not been a universal finding (Lofsness *et al*, 1987). Profound thrombocytopenia with haemorrhagic manifestations is occasionally seen (Clarke & Davies, 1964; Goldstein & Porter, 1969; Radel & Schorr, 1963).

In the current study male cases had significantly lower haemoglobin values, than male controls, at diagnosis and for up to a month later. In females median Hb levels were lower than in controls at all time points, although this only achieved borderline significance in students seen between 15 and 30 days after diagnosis. Haemolytic anaemia is a recognised complication of acute IM, thought to occur in 0.5-3% of cases, usually in the first three weeks after diagnosis (Johannsen *et al*, 2005). None of the cases in the current study who had a FBC taken at the time of diagnosis appeared to have significant haemolysis with only one male case having a Hb value below 130 g l^{-1}, the lower limit of the normal range for males supplied by the Haematology Laboratory, however, this excludes a student admitted to hospital with jaundice whose Hb at diagnosis is unknown.

Abnormality of one or more of the liver function tests was common, affecting 73% of students reviewed within one month of diagnosis. This in keeping with the 83% prevalence noted by Finkel *et al* (1964) and 58% noted by Grotto *et al* (2003). None of the controls had elevation of ALT or ALKP. Elevation of GGT occurred in both cases and controls but was significantly more common in cases, with only one control having more than a trivial elevation of GGT. Amongst cases reviewed within two weeks of diagnosis 41% had ALT levels more than twice the upper limit of normal and in 19% the elevation was more than 5 times the upper limit of normal. There was no significant difference in the proportion of cases (22%) or controls (17%) that had an elevated bilirubin. A similar figure of 21% was

described by Finkel *et al* (1964) in their IM cases although this study did not have a healthy control group.

An increased viral load at diagnosis was not generally associated with features that would suggest more severe clinical disease. Exceptions to this were 4 students with vomiting as part of their IM presentation, and a trend towards significant associations with both an exercise tolerance of less than 100m, and being unable to leave the house at the time of the most severe symptoms. The failure to find an association between viral load and severe clinical illness is in keeping with the findings of Silins *et al* (2001) who found that the EBV viral load in IM and cases undergoing asymptomatic seroconversion was similar. In contrast, Balfour *et al* (2005) did find an association between whole blood viral load and more severe illness, although this was based on an unvalidated scoring system, that ascribed a numerical score (0-3) for the extent of limitation of physical activity and for the intensity of pain / symptoms.

The raised lymphocyte count in IM was composed of elevations of $CD8^+$ T-cells, NK cells and γδ T-cells without any significant change in the numbers of $CD4^+$-T-cells or B-cells. The total lymphocyte count remained significantly above that of the control students for up to 15 days, whereas the elevation of γδ T-cells persisted at all time periods during convalescence. NK cell numbers were elevated at all time points up to 5 months, which was significant for samples taken at diagnosis, in the first month, and between two and four months after diagnosis. Two previous reports have documented raised γδ T-cells at the time of diagnosis in IM; one of these also repeated the observations after 4 weeks finding that γδ T-cells remained significantly elevated when compared with the control group (De Paoli *et al*, 1990; Hassan *et al*, 1991). The current results show a long lasting elevation of this lymphocyte subset together with prolonged NK cells numbers up to 4 months.

Whilst the exact role of γδ T-cells in IM is unknown it is interesting to speculate on their possible functions as these lymphocytes link the innate and adaptive arms of the immune response. When acting as part of the innate immune system γδ T-cells may lyse target cells and release cytokines and chemokines in response to recognition of non-peptide antigens, but in addition they can function as conventional αβ T-cells recognising peptide antigens, and more recently a role in antigen presentation, at least in vitro, has been reported (Brandes *et al*, 2005; Kaufmann, 1996; Welsh *et al*, 1997). A role for γδ T-cells has also been proposed in the resolution of inflammatory states and in anti-cancer surveillance has been proposed (Chen & Letvin, 2003; Déchanet *et al*, 1999; Ferrarini *et al*, 2002; Gao *et al*, 2003). A murine study of HSV infection detected γδ T-cells specific for the viral gp gI, a glycoprotein

which, with gE, forms a viral Fc receptor that binds IgG, facilitating viral cell-cell spread (Johnson *et al*, 1992; Roizman *et al*, 2007). Thus the γδ T-cells in this model targeted a viral immune evasion mechanism.

It is clear from the current observations of γδ and NK cells, and the work of others, that IM has prolonged, possibly permanent, effects on the immune system. Acute EBV infection when manifest as IM, results in massive alterations in lymphocyte specificities with up to 44% of all $CD8^+$ T-cells being specific for a single EBV epitope, and huge increases in lymphocyte numbers, particularly the $CD8^+$ population (Callan *et al*, 1998; Macallan *et al*, 2003). Although the total numbers of $CD8^+$ cells subside quickly over 2 to 3 weeks there remain long lasting perturbations of the $CD8^+$ T-cell population with an increased proportion of cells expressing intracellular IFN-γ at six months (Attarbaschi *et al*, 2003). Tan *et al* studied donors both with and without a history of IM and found up to 5% of $CD8^+$ cells were specific for a single EBV epitope many years after primary infection (Tan *et al*, 1999). Sauce *et al* have observed long lasting defects in EBV-specific $CD8^+$ T-cell responses to IL-7, lasting 2 or more years after IM, and seemingly permanent reductions in IL-15 α receptor expression on $CD4^+$ and $CD8^+$ T-cells and CD56+NK cells following IM (Sauce *et al*, 2006). These, or other yet to be described, immunological abnormalities may contribute to the pathogenesis of Hodgkin's lymphoma, known to occur at an increased frequency for at least 10 years following IM (Hjalgrim *et al*, 2003), further strengthening the case to prevent symptomatic primary EBV infection in populations at risk.

Impact on Academic and Social Activities

One of the objectives of the current study was to document the effects of IM on the daily activities of the students, particularly their studies but also on their domestic and social lives. The illness, particularly when its onset was in term time, had a profound effect on studies with a median loss of 10 hours of timetabled university classes per student, and significantly reduced personal study time resulting in an estimated overall loss of study time of approximately 48 hours per student. Female students missed significantly more university classes (median of 17 hours) than males (median 2 hours) and were more likely to discontinue their studies completely (n=5 versus none). The reason for the greater impact on female students is not known. Some viral illnesses, notably rubella and parvovirus infection, are known to exert more severe morbidity in women (Brown, 2005; Gershon, 2005). It is also known that women have a greater lifetime risk of psychiatric illness than men (Afifi, 2007), and is possible that females are more vulnerable to negative cognitions after IM (e.g. feelings of low mastery, and more rumination or worry).

The severity of the initial disability predicted the quantity of university classes missed, such

that students who could not wash and dress at the worst point of their illness, or that were unable to leave their house, or reported reduced walking distance all missed significantly more classes than cases who did nor report these symptoms. The initial numerical score that cases attributed to their fatigue correlated significantly in both genders with the subsequent number of university classes missed. The majority of IM cases (78%) reported an impaired ability to concentrate recording a median concentration span of 10 minutes at the time of their most severe symptoms whilst controls reported a concentration span of 40 minutes. The perception of impaired concentration was not affected by gender but was significantly associated with the onset of symptoms in term time. There was evidence that students prioritised their academic work over exercise and other social activities after diagnosis. The total study hours (personal and timetabled) were significantly reduced in cases when compared with controls for a period of only 15 days after diagnosis (although the median was somewhat reduced until four months), whereas, significant reductions in hours of exercise persisted for up to 2 months, and hours spent on social activities other than exercise were significantly reduced when compared with controls 5 or more months after diagnosis. Hypersomnia is a recognised feature of IM and cases reported increased hours of sleep compared with controls; a pattern that persisted at 5 months after diagnosis. Non-exercise related social activities were severely curtailed by IM and were significantly reduced compared with controls at 5 or more months after diagnosis, at this stage cases spent 4 hours per week less than controls on social activities, which is in keeping with their reported requirement for additional sleep.

IM cases reported significantly fewer hours of exercise than controls for up to 2 months after diagnosis. Whereas cases and controls reported spending similar amounts of time attending university, studying and socialising, the reported hours of vigorous exercise per week prior to the onset of IM was significantly greater in cases than that reported by the control students. It is not clear why exercise alone should be subject to a particular recall bias, and therefore the possibility exists that higher levels of exercise may itself be a risk factor for IM. Interestingly, a significant positive correlation between fatigue duration and levels of pre-morbid exercise hours was found in male cases. At visits later than 5 months after diagnosis however the amount of exercise undertaken by cases and controls did not differ significantly. The possible explanations for this might be that the cases overstated their pre-morbid exercise hours, or that even though they said that they had recovered they had not taken up their previous exercise regimes or that the numbers of subjects analysed are inadequate to show a significant difference. That cases, even ones that said they no longer felt fatigued, had not regained their previous exercise pattern by 5 or more months after their illness would

support the idea that subtle effects of IM persist for long periods after the acute illness.

Fatigue

Fatigue was undoubtedly the most long lasting of IM-related symptoms. In other studies of fatigue following IM there was a marked gender difference with females experiencing more protracted symptoms (Buchwald *et al*, 2000; Candy *et al*, 2003; Petersen *et al*, 2006; White *et al*, 2001). Buchwald *et al* (2000) found that a greater number of life events in the preceding six months, and greater family support, also predicted ongoing fatigue at six months after diagnosis. Petersen *et al* (2006) found fatigue after IM was associated with premorbid mood disorders. Interestingly in a primary care study of post-infectious fatigue, where only 1% of participants had glandular fever and most had prior sore throat or other upper respiratory tract infections, there was no excess of females in the fatigued group, and instead a link with previous fatigue and previous psychological disorder was found (Wessely *et al*, 1995). In the present study 23% of participants were still fatigued at six months a proportion somewhat closer to the 12% reported by Buchwald *et al* (2000) than the 40% found by White *et al* (1998). Overall the median duration of fatigue in Edinburgh students was 70 days, with a median duration of 125 days in females, and 54 days in males. Fatigue duration was significantly correlated with the number of timetabled classes missed by both genders overall, and in females. In line with the work of White *et al* (2001) and Buchwald *et al* (2000) students that recalled a stressful event prior to the onset of IM were also at greater risk of ongoing fatigue at 6 months after diagnosis.

Fatigue, along with the other symptoms of IM, is generally assumed to be caused by cytokines released by the large numbers of circulating, activated, $CD8^+$ T-cells typically found in the acute disease (Attarbaschi *et al*, 2003; Callan *et al*, 1998; Foss *et al*, 1994; White *et al*, 2001). In this study no association was found between total numbers of peripheral blood lymphocytes, CD4+, CD8+ or NK lymphocyte subsets and fatigue severity or duration. In males, but not in females, there was some evidence that a higher monocyte ($p=0.033$) and total lymphocyte ($p=0.064$) count at diagnosis was associated with a greater number of hours missed from university classes.

Some markers of functional impairment in acute IM were also significantly related to fatigue duration, especially in females, including inability to leave home and inability to walk 100m at the worst point of their illness. White *et al* (2001) also found that "days in bed at onset" predicted an increased risk of fatigue at 2 months after diagnosis. In the Edinburgh students the initial numerical score that cases attributed to their fatigue showed a significant correlation with the duration of subsequent fatigue, and scores were higher in female rather than male cases. The association between score and subsequent fatigue duration was not

significant when the genders were analysed separately, suggesting that the fatigue score was a stronger predictor of missed university classes than fatigue duration.

Candy *et al* reported that patients with IM often recall being advised to rest by their general practitioner, however, beyond the most acute symptoms this may unhelpful (Candy *et al*, 2005). A very early study of predominantly male (91%) students with IM demonstrated that the group allocated to bed rest suffered from significantly more "prolonged fatigue and debility" than the group allowed to mobilise freely (Dalrymple, 1964). A more recent pilot study significantly reduced the incidence of fatigue 6 months after IM by providing a brief individualised face to face session involving a plan for graded activity and lifestyle advice (Candy *et al*, 2004).

Strain Typing

In the LCS, using PBMC collected at recruitment, type 1 EBV was detected in 483 (77%), type 2 was present in 108 (17%) and both types were detected in 34 (5%). In the case control study forty two isolates were type 1 strains (88%), 3 were type 2 (6%) and a further 3 (6%) contained both type 1 and type 2 sequences. It can be seen that there is a higher proportion type 1 strains amongst the IM cases than in the cohort as a whole, however, this did not attain statistical significance[3]. It might be expected that IM would be caused by type 1 strains more often than type 2 in view of the differing in vitro behaviour of the two types, whereby EBV-1 shows greater ability to stimulate B cell activation and immortalisation (Rickinson *et al*, 1987). Gratama and Ernberg (1995) previously observed that 7/7 EBV isolates from IM cases were type A (the old designation for EBV type 1). A larger study which included the cases documented in the present work, together with additional IM cases, has demonstrated a significant excess of type 1 in the aetiology of IM, compared with the background distribution of EBV types in the same population (Crawford *et al*, 2006).

The current study has demonstrated that cases and their friends or household contacts were much more likely to have differing EBV isolates than cases and their partners who had indistinguishable isolates in 9/11 (82%) pairs. As the number of repeats at each locus studied is finite, it is possible that isolates could be categorised as indistinguishable but in fact be different had additional typing been performed. The observation of strain sharing by sexual partners is further complicated by recent work suggesting that primary infection may involve the transmission of multiple strains (Sitki-Green *et al*, 2003; Sitki-Green *et al*, 2004). In two instances in the current study, (an IM case and his girlfriend, and another case and their flatmate) there was a match at one locus and a mismatch at the other. These were

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[3] Fisher exact test of the heterogeneity of proportions (personal communication C. Higgins)

classified as mismatches for the purposes of the study but could represent the detection of mixed populations.

Gender Bias

Females were more likely to participate in all parts of this investigation into EBV infection in Edinburgh University Students. Of the 2006 students recruited to the LCS 63% were female, which is greater than the proportion of females (53%[4]) undertaking four year courses in the years 1999 and 2000, and is closer to the proportion of females that registered with the UHC (59%[5]) where the recruitment took place. The higher proportion of females recruited to the LCS would seem to reflect both the higher health care seeking behaviour of women (Rickards *et al*, 2004) and the greater participation of women in studies of this type (Candy *et al*, 2003; Stewart-Brown *et al*, 2000; White *et al*, 2001). Amongst the 241 seronegative students that returned for follow-up after three years 62% were female, which represents an excess as only 54% of the seronegative students were female at recruitment.

The gender bias was less pronounced in recruitment to the case control study where 53% of participants were female. The higher proportion of males in the case control study (47%) probably reflects the higher seronegative rate in male students entering university, and is close to the percentage of serology tests consistent with acute IM at the UHC that occurred in males (49%)[6]. The recruitment to this part of the study was poorer as it was not immediate and relied on the GPs in the UHC to give IM cases an information leaflet, seek permission for the student to be approached, obtain a telephone number and then fax this information. None-the-less despite this circuitous process the rate of recruitment was in line with the 45-49% achieved in other studies of student health in primary care (Rea *et al*, 2001; Stewart-Brown *et al*, 2000).

Female cases completed a greater number of follow-up visits than males (mean 2.9 and 2.1 respectively) and were more likely to attend for a final follow-up visit (63% vs. 54%, p=0.710). As noted above females are more likely to participate in research studies than males, however, having been recruited their greater likelihood to attend more follow-up visits may reflect their longer duration of symptoms. Reasons for non-attendance at a final

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[4] Personal communication, Andrew Quickfall, Governance and Strategic Planning, University of Edinburgh (see Appendix 12.3)

[5] Personal communication, Nadine Harrison, University Health Centre

[6] Analysis of the diagnostic serology undertaken for the University Health Centre in the years 2000-2003 showed that of positive tests, consistent with an IM diagnosis, 49% were in males, and 51% in females.

review visit after 5 months included being unable to contact the student by email or telephone, the student having left the university through illness or completion of their degree, and the student declining further follow-up.

10 Conclusions

Whilst EBV may be mainly transmitted in saliva rather than in genital secretions the current investigation has demonstrated that sexually active students are much more likely to have acquired EBV, and that students presenting with IM are likely to be sexually active. This has implications for their healthcare as a whole as they may have additional requirements including contraception and sexual health screening, for infections known to have a high prevalence in young adults, including *Chlamydia trachomatis*. The study has demonstrated long lasting fatigue and functional impairment (academic, social and physical exercise) following IM indicating that primary preventative strategies, particularly immunisation when it becomes available, and tertiary preventative strategies to prevent disability, such as the graded activity and lifestyle management approach piloted by Candy *et al* (2004), may reduce morbidity in this population. Females and those recalling a stressful event prior to the onset of IM are at particular risk of adverse outcome following IM.

11 Further Work

The current study demonstrated an association between the numerical score ascribed by students to the severity of their initial fatigue and subsequent adverse outcomes including fatigue duration and the number of university classes missed. The prognostic accuracy of this simple tool deserves further exploration as a means to predict poor outcomes in IM. Incorporating behavioural measures to encourage activity and prevent physical deconditioning, thought to worsen fatigue, as piloted by Candy *et al* (2004), should be further explored to reduce IM associated morbidity.

IM appears to be associated with failure of course completion in some students. Exit interviews of all students leaving university without completing their degree could be offered to investigate the extent of this effect. It is possible that IM may adversely effect the class of degree that students achieve. This could be investigated by prospectively assessing academic performance before and after IM.

The role of the γδ T-cells in IM is not known. The current study has demonstrated that they persist in raised numbers long into the convalescent period. Recent work has demonstrated

that these cells, in addition to linking the innate and adaptive immune responses, can also act as antigen presenting cells. The role and specificity of these interesting cells could be further studied.

Type 1 EBV shows greater ability to stimulate B-cell activation and immortalisation and has now been shown to be over-represented in the aetiology of IM as compared with type 2 EBV (Crawford *et al*, 2006; Rickinson *et al*, 1987). It is not known if there are differences in the viral load, clinical manifestations or immune parameters in IM caused by the different types, although preliminary investigation of the lymphocyte count at the time of diagnosis in this study suggests that IM caused by type 1 is associated with a higher lymphocyte count than IM caused by type 2 strains (Appendix 12.2).

There is some evidence to suggest that the epidemiology of herpes viruses, particularly HSV and EBV, is changing, with acquisition being delayed into teenage years (Morris *et al*, 2002; Morris & Edmunds, 2002; Scoular *et al*, 2002; Takeuchi *et al*, 2006; Vyse *et al*, 2000). This is likely to result in an increased proportion of symptomatic illness and increased rate of complications and hospitalisations (Auwaerter, 1999; Halevy & Ash, 1988; Horwitz *et al*, 1983; Morris & Edmunds, 2002; Tattevin *et al*, 2006). This study has demonstrated the importance of IM as a cause of morbidity in students and disruption to university studies, however, the impact of IM on adolescent school children and their studies has not been addressed. The current study has also shown a high annual rate of EBV seroconversion. The rate of annual seroconversion in adolescent schoolchildren would need to be determined in order to offer immunisation at the optimal age.

Study of sexual behaviour has indicated a progressive fall in the age at first sexual intercourse in the UK during the 1970s and 1980s (Wadsworth *et al*, 1993; Wellings *et al*, 1994; Wellings *et al*, 2001) with 30% of male 16-19 year olds and 25% of females reporting first sexual intercourse before the age of 16. The current study has demonstrated a strong linkage with sexual behaviour, it therefore follows that the epidemiology of IM may be affected by changing patterns of sexual behaviour and increasingly become an illness of adolescent schoolchildren as well as college students. It could be anticipated that industrialisation and development in other parts of the world where IM was previously rare, for example Brazil, Thailand and Malaysia, would result in an increase in the population rate of IM due to delays in the age of primary infection, but as yet no published reports have appeared. Future seroepidemiological studies and the schedule of any EBV immunisation programme needs to take account of changes in the age of primary infection and sexual behaviour.

12 Appendix

12.1 Questionnaires and Data Collection Sheets

EDINBURGH STUDENT QUESTIONNAIRE

YEAR 1

CONFIDENTIAL

ID Number:

Date of questionnaire completion	☐☐ Day	☐☐ Month	☐☐ Year

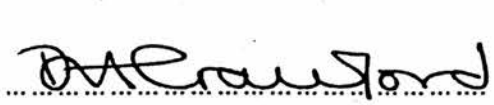
Introduction

We are conducting a research study in Edinburgh University students on the ways in which personal and family background and life-styles relate to getting glandular fever. The information sheet you have been given describes the study, why we believe it is important and what you would need to do. We hope that you will be willing to take part. If you have any questions please speak to one of the attendant staff, or Dr Sheena Sutherland, Department of Medical Microbiology, University of Edinburgh, Medical School, who is not directly involved in the study.

Thank you, in anticipation, for your help.

Dorothy H Crawford
Professor of Medical Microbiology
University of Edinburgh

Dr Nadine Harrison
University Health Service
University of Edinburgh



THIS QUESTIONNAIRE IS ANONYMOUS SO NONE OF THE ANSWERS YOU GIVE CAN BE PASSED TO THE UNIVERSITY OR ANYONE ELSE.

YOU ARE UNDER NO OBLIGATION TO TAKE PART IN THIS STUDY OR TO FILL IN THIS QUESTIONNAIRE NOW – IF YOU WISH WE CAN MAKE ARRANGEMENTS FOR YOU TO COME BACK ANOTHER TIME.

ID Number:

1. *Personal History and Family Background*

This information will be used to examine the association between spread of infection and living circumstances, family size, position in the family etc.

1.1 Where were you born?:

Town: .....

Country: .....

1.2 When were you born?: ☐☐ day ☐☐ month ☐☐ year

1.3 What is your ethnic group?

- White ☐ 1
- Black ☐ 2
- S. Asian ☐ 3
- Chinese ☐ 4
- Other ☐ 5 please specify .....

1.4 What religion were you brought up in?

- Christian ☐ 1
- Jewish ☐ 2
- Muslim ☐ 3
- Hindu ☐ 4
- Sikh ☐ 5
- Other ☐ 6 please specify .....
- None ☐ 7

1.5 Are you: male ☐ 1 female ☐ 2

1.6 Are you a: **twin** ☐1 **triplet** ☐2 **or** **singleton-born** ☐3 **?**

If twin, is your co-twin **male** ☐1 **or** **female** ☐2

If triplet, what are the sexes of your co-triplets ..... ?

If you are a twin or triplet, we would like to contact your siblings for this study, to ask them the same questions we have asked you. It would of course be entirely their choice whether they wished to participate. If you are agreeable to us contacting them, please could you enter their name and address:

.....

.....

.....

1.7 Do you have any brothers or sisters (including half or step brothers or sisters)?

Yes ☐1 No ☐2 **If yes:**

Relationship:	**Age (years):**	**Lived with until you were what age? (Years)**
1. Brother ☐ Sister ☐		
2. Brother ☐ Sister ☐		
3. Brother ☐ Sister ☐		
4. Brother ☐ Sister ☐		
5. Brother ☐ Sister ☐		
6. Brother ☐ Sister ☐		

(please continue at end of questionnaire if required)

1.8 What were your parents' occupation(s) when you were a child (if many, state the longest)?

Father's occupation: .....

Mother's occupation: .....

1.9 Are you:	**tick:**
Married?	☐1
Widowed?	☐2
Divorced or Separated?	☐3
Cohabiting (living with partner)?	☐4
In a relationship?	☐5
Single?	☐6

1.10 Do you have any children? Yes ☐1 No ☐2 **If yes:**

Sex:		**Date of Birth:**		
Male ☐1	Female ☐2	☐☐ Day	☐☐ Month	☐☐ Year
Male ☐1	Female ☐2	☐☐ Day	☐☐ Month	☐☐ Year
Male ☐1	Female ☐2	☐☐ Day	☐☐ Month	☐☐ Year
Male ☐1	Female ☐2	☐☐ Day	☐☐ Month	☐☐ Year

2. *Questions About Crowding and Living Circumstances*

This information will be used to determine whether close contact is a risk factor for early infection.

2.1 As a child, did you share a bedroom?:	
Never, or almost never	☐1
Occasionally, but not continuously (eg when there were visitors)	☐2
Continuously, from ages:	☐☐ to ☐☐ years
If continuously, with whom did you share?	Relationship: Brother ☐1 Sister ☐2 Other ☐3 please specify .....
	Older than you? ☐1 or younger than you? ☐2
	Or same age (twin)? ☐3
If shared continuously on more than one occasion, please give details	.....

2.2 For each school you attended for a year or more, please give details:

From (Age)	To (Age)	(tick:) Day attendee	(tick:) Boarder	Approx. no of children in the school	Age range of school
1.		☐	☐		
2.		☐	☐		
3.		☐	☐		
4.		☐	☐		
5.		☐	☐		
6.		☐	☐		
7.		☐	☐		

(please continue at end of questionnaire if required)

2.3 Where have you lived? Please list each village/town you have lived in for a year or more, starting with where you were born:

From (Age, years)	To (Age, years)	Village/Town	Country	Approx no of rooms in house (living rooms and bedrooms) (average if this varied)*	No of people living in the house/flat (average if this varied)*
1.					
2.					
3.					
4.					
5.					
6.					
7.					

(please continue at end of questionnaire if required)

* If you lived in an institution such as a hall of residence, please say so instead of stating the numbers of rooms and people.

2.4 Have you ever lived in a developing or tropical country? Yes ☐1 No ☐2

If yes:

Where? .....

From age ☐☐ to ☐☐ years

*3. **Medical Background***

Past and present medical and growth history may be important in the susceptibility to infection.

3.1. What was your weight at birth?

Kilograms ☐.☐☐ **or** ☐☐☐☐ grammes **or** lbs☐☐ oz☐☐

If birthweight not known, were you at birth:

small ☐1 average ☐2 large ☐3 don't know ☐4

Were you breast fed? yes ☐1 no ☐2 don't know ☐8

3.2 Height/Weight:

Current height ☐ ft ☐☐ ins **or** ☐.☐☐ metres

Current weight ☐☐ st ☐☐ lbs **or** ☐☐.☐ kg

3.3 At primary school were you: **tick:**

Tall for your age? ☐1

Short for your age? ☐2

About average? ☐3

3.4 If female, **tick:**

- age of first period ☐☐ years **or** never started ☐
- age regular periods started ☐☐ years **or** never became regular ☐

3.5 Have you been immunised against: **tick:**

	Yes	No	Don't know
Polio	☐1	☐2	☐8
Measles	☐1	☐2	☐8
Mumps	☐1	☐2	☐8
Rubella	☐1	☐2	☐8
TB (BCG)	☐1	☐2	☐8
Typhoid	☐1	☐2	☐8
Tetanus	☐1	☐2	☐8
Triple (Diptheria/Tetanus/Pertussis)	☐1	☐2	☐8
Hepatitis B	☐1	☐2	☐8

Other, please state .....

3.6 Have you had (at any age) any of the following illnesses? If so, at what age?

	tick: Yes	Probably Yes	**tick:** No	Probably No	If yes or probably yes, approx age (years)
Measles	☐1	☐2	☐3	☐4	
Mumps	☐1	☐2	☐3	☐4	
German measles	☐1	☐2	☐3	☐4	
Whooping cough	☐1	☐2	☐3	☐4	
Glandular fever (infectious mononucleosis)	☐1	☐2	☐3	☐4	
Chickenpox	☐1	☐2	☐3	☐4	
Hepatitis A	☐1	☐2	☐3	☐4	
Hepatitis B	☐1	☐2	☐3	☐4	

3.7 Do you have, or have you had, any of the following allergies? If yes, was this diagnosis confirmed by a doctor? Were you treated with any prescribed medications?

	Tick:		Doctor diagnosed?:		Were you ever treated with: (tick)		
	Yes	No	Yes	No	Drugs	Creams	Inhalers
Eczema	☐1	☐2	☐1	☐2	☐1	☐2	☐3
Hay fever	☐1	☐2	☐1	☐2	☐1	☐2	☐3
Asthma	☐1	☐2	☐1	☐2	☐1	☐2	☐3
Drug or food allergies, specify	☐1	☐2	☐1	☐2	☐1	☐2	☐3
Other allergies, specify	☐1	☐2	☐1	☐2	☐1	☐2	☐3

Sexual contacts might be relevant to whether people get infections.

3.8 Have you ever been sexually active? Yes ☐1 No ☐2

If no: proceed to question 3.10

If yes:

Age of first sexual intercourse ☐☐ years

Number of sexual partners, ever ☐☐

Number of sexual relationships of a year or more duration ☐☐

		tick:
Use of condom:	always	☐1
	occasionally	☐2
	never	☐3
	not applicable	☐7
Were your partner(s):	same sex?	☐1
	opposite sex?	☐2
	both?	☐3

3.9 Have you ever had a sexually transmitted disease? Yes ☐ 1 No ☐ 2

If yes, at what age? (first occurrence if several) ☐☐ years

If yes, which of the following have you had:

	tick:
Gonorrhoea	☐
Syphilis	☐
NSU (chlamydia)	☐
HIV	☐
Genital warts	☐
Genital herpes	☐

3.10 Have you had any other serious or longstanding illnesses or operations: (including all illnesses for which admitted to hospital)?

Yes ☐ 1 No ☐ 2 **If yes, please list:**

	Illness/Operation	Age at onset / operation
1.		
2.		
3.		
4.		

(please continue at end of questionnaire if required)

3.11 Have you ever had a blood transfusion? Yes ☐ 1 No ☐ 2

If yes, on how many different occasions? ☐

When was the first time? 1 9 ☐☐ (year)

When was the most recent time? 1 9 ☐☐ (year)

3.12 Are you using any medication (including oral contraceptives and inhalers) now?

Yes ☐ 1 No ☐ 2 **If yes, please state which :**

1.
2.
3.
4.
5.
6.

(please continue at end of questionnaire if necessary)

3.13 Mental illnesses?

Have you ever had a mental/psychological illness/problem: Yes ☐1 No ☐2

If yes, was it:

Depression requiring medical treatment ☐
Drug overdose ☐
Anorexia ☐
Bulimia ☐
Schizophrenia ☐

Other (please state) .....

3.14 Have you experienced a major life event in the last year? Yes ☐1 No ☐2

For example:				month	year
Has your parent, child, sibling or spouse died?	Yes ☐1	No ☐2	If yes when?	☐☐	☐☐
Have you broken off a marriage or steady relationship?	Yes ☐1	No ☐2	If yes when?	☐☐	☐☐
Have you had a major financial crisis?	Yes ☐1	No ☐2	If yes when?	☐☐	☐☐
Have you had major problems with your studies/exams?	Yes ☐1	No ☐2	If yes when?	☐☐	☐☐

Other, please state: .....

date:.....

4. *Smoking, Drinking and Exercise*

Social habits like smoking, and drinking alcohol may effect your susceptibility to infection.

4.1 Smoking:

Do you, or have you ever, regularly smoked tobacco (ie smoked at least 1 cigarette or cigar or pipe per day, on average, for at least 1 year)? Yes ☐1 No ☐2

If yes, how old were you when you started to smoke regularly? ☐☐ years

During the period when you were a regular smoker, how much, on average, did you smoke?

cigarettes / day ☐☐

cigars / day ☐☐

hand rolling tobacco oz / week ☐☐

pipe tobacco oz / week ☐☐

Have you now stopped smoking? Yes ☐1 No ☐2

If yes, how old were you when you stopped? ☐☐ years

4.2 Alcohol:

Do you or have you drunk alcohol regularly (once / week or more for at least a year)?:

Yes ☐1 No ☐2

If yes, at what age did you start to drink regularly? ☐☐ Years

Do you still drink regularly? **tick:** Yes ☐1 No ☐2

During the period when you were a regular drinker, how much, on average, did you drink?

Beer or cider (pints) ☐ per day **or** ☐☐ per week

Wine (glasses) ☐ per day **or** ☐☐ per week

Spirits (tots) ☐ per day **or** ☐☐ per week

4.3 Exercise: Since age 11, have you regularly (once / week or more) engaged in active sports or other active exercise sufficient to raise a sweat or get you out of breath?

Yes ☐ 1 No ☐ 2

If yes, number of hours / week on average:-

aged 11-16 years	☐☐ summer	☐☐ winter
aged over 16 years	☐☐ summer	☐☐ winter

THANK YOU VERY MUCH FOR HELPING US WITH THIS SURVEY

We appreciate your help, and hope that you have felt able to answer the questions without too much trouble. If there is anything you would like to add or comment upon, please write it below:-

_____

_____

_____

_____

EDINBURGH STUDENT QUESTIONNAIRE

GLANDULAR FEVER STUDY

CONFIDENTIAL

ID Number:	Male ☐ Female ☐		
Date of Birth	☐☐ Day	☐☐ Month	☐☐ Year
Date of Filling in Questionnaire	☐☐ Day	☐☐ Month	☐☐ Year

Introduction

You may recall that when you first arrived at Edinburgh University you filled in a questionnaire and gave a blood sample as part of a study investigating the ways in which your lifestyle and background relate to your risk of developing glandular fever. Later on you should have received the result of this blood test from us. Now that you have been at University for 3 years, we would appreciate it if you would fill in a further questionnaire and allow us to take some more blood. Once again your replies will be treated confidentially. The information will not be disclosed to the University or others. Your participation is voluntary and whether you participate or not will make no difference to your medical care. If you feel that any of the questions are intrusive, please feel free to pass on to the next without answering.

If you wish to discuss any aspects of the study further, please contact Dr. Tanzina Haque, Basic & Clinical Virology IDG, R(D)VS, Tower Block, Summerhall, Edinburgh EH9 1QH (tel. 0131 650 7941), who is not directly involved in the study.

Thank you, in anticipation, for your help.

Dorothy H Crawford
Professor of Medical Microbiology
University of Edinburgh

Dr Nadine Harrison
University Health Service
University of Edinburgh

....

1. *QUESTIONS ABOUT YOUR STUDIES*

1.1 When did you start at Edinburgh University?

☐☐ Month ☐☐ Year

1.2 What course are you undertaking?

Please specify .....

2. *PERSONAL AND FAMILY DETAILS*

2.1 If you were regularly looked after outside your own home before attending primary school, what were the arrangements for looking after you, and from approximately what age did these apply?

Tick more than one box if appropriate.

	Yes	Age started (years)	No	Don't know
Nursery school or reception class	☐	☐	☐	☐
Other day care centre	☐	☐	☐	☐
At childminder's home	☐	☐	☐	☐
Other outside home – please specify	☐	☐		

.....

2.2 Do you have any children? Yes ☐1 No ☐2 **If yes:**

Sex:		Date of Birth:		
Male ☐1	Female ☐2	☐☐ Day	☐☐ Month	☐☐ Year
Male ☐1	Female ☐2	☐☐ Day	☐☐ Month	☐☐ Year
Male ☐1	Female ☐2	☐☐ Day	☐☐ Month	☐☐ Year
Male ☐1	Female ☐2	☐☐ Day	☐☐ Month	☐☐ Year

3. *QUESTIONS ABOUT YOUR LIVING CIRCUMSTANCES*

3.1 Please list each residence you have lived in for 3 months or more since coming to Edinburgh University (excluding your parents' home):

1ST RESIDENCE:

(a) from ☐☐ / ☐☐ (M / Y) to ☐☐ / ☐☐ (M / Y)

Village/town/city ..... Country .....

Type of accommodation: please tick:

Halls of residence	☐
House	☐
Flat	☐
Other	☐ please specify .....

With how many adults and how many children on average did you share the following Rooms (exclude yourself)?:

	Adults	Children
Kitchen	☐	☐
Bathroom	☐	☐
Bedroom	☐	☐
Living room	☐	☐

3.2 2nd RESIDENCE:

(b) from ☐☐ / ☐☐ (M / Y) to ☐☐ / ☐☐ (M / Y)

Village/town/city ..... Country .....

Type of accommodation: please tick:

Halls of residence	☐
House	☐
Flat	☐
Other	☐ please specify .....

With how many adults and how many children on average did you share the following rooms?:

	Adults	Children
Kitchen	☐	☐
Bathroom	☐	☐
Bedroom	☐	☐
Living room	☐	☐

3.3 **3rd RESIDENCE:**

(c) from ☐☐ / ☐☐ (M / Y) to ☐☐ / ☐☐ (M / Y)

Village/town/city ..... Country .....

Type of accommodation: **please tick:**

Halls of residence	☐
House	☐
Flat	☐
Other	☐ please specify .....

With how many adults and how many children on average did you share the following rooms?:

	Adults	Children
Kitchen	☐	☐
Bathroom	☐	☐
Bedroom	☐	☐
Living room	☐	☐

3.4 **4th RESIDENCE:**

(b) from ☐☐ / ☐☐ (M / Y) to ☐☐ / ☐☐ (M / Y)

Village/town/city ..... Country .....

Type of accommodation: **please tick:**

Halls of residence	☐
House	☐
Flat	☐
Other	☐ please specify .....

With how many adults and how many children on average did you share the following rooms?:

	Adults	Children
Kitchen	☐	☐
Bathroom	☐	☐
Bedroom	☐	☐
Living room	☐	☐

3.5 **5th RESIDENCE:**

(b) from ☐☐ / ☐☐ (M / Y) to ☐☐ / ☐☐ (M / Y)

Village/town/city ..... Country .....

Type of accommodation: **please tick:**

Halls of residence	☐
House	☐
Flat	☐
Other	☐ please specify .....

With how many adults and how many children on average did you share the following rooms?:

	Adults	Children
Kitchen	☐	☐
Bathroom	☐	☐
Bedroom	☐	☐
Living room	☐	☐

3.6 **6th RESIDENCE:**

(b) from ☐☐ / ☐☐ (M / Y) to ☐☐ / ☐☐ (M / Y)

Village/town/city ..... Country .....

Type of accommodation: **please tick:**

Halls of residence	☐
House	☐
Flat	☐
Other	☐ please specify .....

With how many adults and how many children on average did you share the following rooms?:

	Adults	Children
Kitchen	☐	☐
Bathroom	☐	☐
Bedroom	☐	☐
Living room	☐	☐

3.7 If during the last 6 months you have lived in any residences for <u>1 month</u> or more that you have not listed above, please list them here (including parental home):

<u>1ST RESIDENCE</u>:

(b) from ☐☐ / ☐☐ (M / Y) to ☐☐ / ☐☐ (M / Y)

Village/town/city ..... Country .....

Type of accommodation: please tick:

Halls of residence	☐
House	☐
Flat	☐
Other	☐ please specify .....

With how many adults and how many children on average did you share the following rooms?:

	Adults	Children
Kitchen	☐	☐
Bathroom	☐	☐
Bedroom	☐	☐
Living room	☐	☐

3.8 <u>2nd RESIDENCE</u>:

(b) from ☐☐ / ☐☐ (M / Y) to ☐☐ / ☐☐ (M / Y)

Village/town/city ..... Country .....

Type of accommodation: please tick:

Halls of residence	☐
House	☐
Flat	☐
Other	☐ please specify .....

With how many adults and how many children on average did you share the following rooms?:

	Adults	Children
Kitchen	☐	☐
Bathroom	☐	☐
Bedroom	☐	☐
Living room	☐	☐

3.9 **3rd RESIDENCE:**

(b) from ☐☐ / ☐☐ (M / Y) to ☐☐ / ☐☐ (M / Y)

Village/town/city ..... Country .....

Type of accommodation: **please tick:**

Halls of residence	☐
House	☐
Flat	☐
Other	☐ please specify .....

With how many adults and how many children on average did you share the following rooms?:

	Adults	Children
Kitchen	☐	☐
Bathroom	☐	☐
Bedroom	☐	☐
Living room	☐	☐

3.10 **4th RESIDENCE:**

(b) from ☐☐ / ☐☐ (M / Y) to ☐☐ / ☐☐ (M / Y)

Village/town/city ..... Country .....

Type of accommodation: **please tick:**

Halls of residence	☐
House	☐
Flat	☐
Other	☐ please specify .....

With how many adults and how many children on average did you share the following rooms?:

	Adults	Children
Kitchen	☐	☐
Bathroom	☐	☐
Bedroom	☐	☐
Living room	☐	☐

4. *MEDICAL BACKGROUND*

4.1 Since coming to University have you been immunised against any of the following?:

Please tick:

	Yes	No	Don't know
Polio	☐ 1	☐ 2	☐ 8
Rubella	☐ 1	☐ 2	☐ 8
TB (BCG)	☐ 1	☐ 2	☐ 8
Typhoid	☐ 1	☐ 2	☐ 8
Tetanus	☐ 1	☐ 2	☐ 8
Hepatitis A	☐ 1	☐ 2	☐ 8
Hepatitis B	☐ 1	☐ 2	☐ 8
Meningitis	☐ 1	☐ 2	☐ 8
Yellow Fever	☐ 1	☐ 2	☐ 8
Japanese B Encephalitis	☐ 1	☐ 2	☐ 8
Rabies	☐ 1	☐ 2	☐ 8
Diphtheria	☐ 1	☐ 2	☐ 8
Other	☐ 1	☐ 2	☐ 8

Other, please state.....

4.2 Since coming to University have you had:

Glandular fever Yes ☐ No ☐

If yes:

Was the diagnosis confirmed by a blood test?

Yes ☐ No ☐

Did you see the glandular fever study doctor?

Yes ☐ No ☐

Did you have any illness with sore throat **and** fatigue for 3 days or more for which you did not seek medical advice?

Yes ☐ No ☐

If so, when? ☐☐ / ☐☐ (M / Y)

☐☐ / ☐☐ (M / Y)

☐☐ / ☐☐ (M / Y)

4.3 **Since coming to University have you had any other physical illnesses that required a visit to your doctor? (excluding visits for vaccinations, contraception)**

Yes ☐ No ☐

If yes, was the visit for:	**Tick:** Yes	**Tick:** No	**Tick:** Time off study (days)
Short illness with full recovery (e.g. 'flu) (Please specify):	☐1	☐2	
(1)			☐
(2)			☐
(3)			☐
(4)			☐
On-going medical condition (e.g. diabetes) (Please specify):	☐1	☐2	
(1)			☐
(2)			☐
(3)			☐
(4)			☐
Other illness (e.g. an operation) (Please specify):	☐1	☐2	
(1)			☐
(2)			☐
(3)			☐
(4)			☐

4.4 **Have you ever had your tonsils out?** Yes ☐1 No ☐2

If so, at what age (in years)? .....

4.5 Since coming to University have you experienced psychological/mental illness? If yes, was this:

	Tick:		Did you receive professional help?	
	Yes	No	Yes	No
Depression	☐1	☐2	☐1	☐2
Drug overdose	☐1	☐2	☐1	☐2
Anorexia	☐1	☐2	☐1	☐2
Bulimia	☐1	☐2	☐1	☐2
Schizophrenia	☐1	☐2	☐1	☐2
Drug/alcohol related problems	☐1	☐2	☐1	☐2
Stress-related problems	☐1	☐2	☐1	☐2

Other, please state .....

4.6 Since coming to University have you experienced any of the following stressful events?

	Tick:		If Yes, When? (date)
	Yes	No	
Your parent, child, sibling or partner was seriously ill or died?	☐1	☐2	☐☐/☐☐ M Y
You broke off a steady relationship or a marriage?	☐1	☐2	☐☐/☐☐ M Y
You had major financial worries?	☐1	☐2	☐☐/☐☐ M Y
You had a pregnancy or termination?	☐1	☐2	☐☐/☐☐ M Y
You had major problems with your studies/exams (e.g. failed exams)?	☐1	☐2	☐☐/☐☐ M Y

Other (please specify) ..... ☐☐/☐☐ M Y

4.7 Since coming to University have you used any medication (including oral contraceptives and inhalers, excluding creams, lotions, drops)? Please state drug type, e.g. antibiotics, steroids.

Yes ☐1 No ☐2 **If yes, please state which and when:**

1.
2.
3.
4.
5.
6.
(please continue at end of questionnaire if necessary)

4.8 (a) Whilst you have been at University have you had contact with anyone who has had glandular fever?

Yes ☐1 No ☐2 If yes, how many? ☐☐

For each contact please record the details below:

First contact

What was your relationship to the person? .....

(b) Do/did you live with this person whilst they had glandular fever or during the 3 months preceding their illness?

Yes ☐1 No ☐2

If yes, was it in a:

House	Yes ☐1	No ☐2
Flat	Yes ☐1	No ☐2
Hall of residence	Yes ☐1	No ☐2

Did you share a:

Bedroom	Yes ☐1	No ☐2
Kitchen	Yes ☐1	No ☐2
Bathroom	Yes ☐1	No ☐2

(c) How often do/did you see them whilst they had glandular fever? Was it:

Daily	Yes ☐1	No ☐2

At least weekly	Yes	☐ 1	No	☐ 2
Less than weekly	Yes	☐ 1	No	☐ 2

4.9 **Second contact**

What was your relationship to the person? .....

(c) Do/did you live with this person whilst they had glandular fever or during the 3 months preceding their illness?

Yes ☐ 1 No ☐ 2

Was it in a:

House	Yes	☐ 1	No	☐ 2
Flat	Yes	☐ 1	No	☐ 2
Hall of residence	Yes	☐ 1	No	☐ 2

Did you share a:

Bedroom	Yes	☐ 1	No	☐ 2
Kitchen	Yes	☐ 1	No	☐ 2
Bathroom	Yes	☐ 1	No	☐ 2

(d) How often do/did you see them whilst they had glandular fever? Was it:

Daily	Yes	☐ 1	No	☐ 2
At least weekly	Yes	☐ 1	No	☐ 2
Less than weekly	Yes	☐ 1	No	☐ 2

4.10 **Third contact**

What was your relationship to the person? .....

(d) Do/did you live with this person whilst they had glandular fever or during the 3 months preceding their illness?

Yes ☐ 1 No ☐ 2

Was it in a:

House	Yes	☐ 1	No	☐ 2
Flat	Yes	☐ 1	No	☐ 2
Hall of residence	Yes	☐ 1	No	☐ 2

Did you share a:

Bedroom	Yes	☐ 1	No	☐ 2
Kitchen	Yes	☐ 1	No	☐ 2
Bathroom	Yes	☐ 1	No	☐ 2

(e) How often do/did you see them whilst they had glandular fever? Was it:

Daily	Yes	☐ 1	No	☐ 2
At least weekly	Yes	☐ 1	No	☐ 2

Less than weekly	Yes ☐1	No ☐2	

4.11 Since you have been at University have you had occurrence of any of the following allergies? If yes, was this diagnosis confirmed by a doctor? Were you treated with any prescribed medications?

	Tick:		Doctor diagnosed?		Were you ever treated with: (tick)		
	Yes	No	Yes	No	Drugs	Creams	Inhalers
Eczema	☐1	☐2	☐1	☐2	☐1	☐2	☐3
Hay fever	☐1	☐2	☐1	☐2	☐1	☐2	☐3
Asthma	☐1	☐2	☐1	☐2	☐1	☐2	☐3
Drug or food allergies (specify)	☐1	☐2	☐1	☐2	☐1	☐2	☐3
Other allergies (specify)	☐1	☐2	☐1	☐2	☐1	☐2	☐3

5. *SMOKING, DRINKING, EXERCISE AND TRAVEL*

5.1 Smoking

Since coming to University have you smoked tobacco (i.e. smoked at least 1 cigarette or cigar or pipe per day, on average)? Yes ☐1 No ☐2

If yes, how much, on average, did/do you smoke?

cigarettes / day ☐☐

cigars / day ☐☐

hand rolling tobacco oz / week ☐☐

pipe tobacco oz / week ☐☐

5.2 Alcohol

Since coming to University have you drunk alcohol?

Yes ☐1 No ☐2

If yes, how much, on average, do you drink? (answer per day or per week, as you feel most representative of your consumption)

Beer or cider (pints) ☐☐ per day **or** ☐☐ per week

Wine (glasses) ☐☐ per day **or** ☐☐ per week

Spirits (tots) ☐☐ per day **or** ☐☐ per week

5.3 Exercise

Since coming to University have you regularly (once a week or more) engaged in active sports or other active exercise sufficient to raise a sweat or get you out of breath for at least 20 minutes?

Yes ☐1 No ☐2

If yes, number of hours / week on average: ☐☐

5.4 Travel

Since coming to University have you travelled outside the UK? Yes ☐1 No ☐2

If yes:
1ST TRIP

Which countries? (list names)
.....

.....

When did you go? ☐☐ / ☐☐
M **Y**

How long was the trip? .....

Did you suffer from travel-related illness? Yes ☐1 No ☐2

If yes, please specify .....

2ND TRIP

Which countries? (list names)

.....

.....

When did you go? ☐☐/☐☐
M Y

How long was the trip? .....

Did you suffer from travel-related illness? Yes ☐1 No ☐2

If yes, please specify .....

3RD TRIP

Which countries? (list names)

.....

.....

When did you go? ☐☐/☐☐
M Y

How long was the trip? .....

Did you suffer from travel-related illness? Yes ☐1 No ☐2

If yes, please specify .....

If you have visited more countries, please continue at end.

6. *SEXUAL RELATIONSHIPS*

Confidentiality:

We are aware that the questions in this section are personal. Your answers will be treated in strict confidence. If you do not feel happy about answering any or all of these questions that is fine, just leave them out and turn to page 20. The notes below are to ensure everyone applies the same meaning to certain terms we use. Please read them carefully before continuing.

NOTES

Genital area
A man's penis or woman's vagina/vulva - that is, sex organs.

(a) Any sexual contact or experience
This is a wider term and can include just kissing or cuddling, but not leading to genital contact or intercourse.

(b) Genital contact NOT involving intercourse
Forms of contact with the genital area NOT leading to intercourse (vaginal, oral or anal) but intended to be sexually stimulating, for example, stimulating by hand.

(c) Oral sexual intercourse
A man's or woman's mouth on a partner's genital area.

(d) Penetrative sexual intercourse
Either vaginal or anal sexual intercourse.

Partner/relationship
People who have had sexual experiences together, of any type (a), (b), (c) and (d) referred to above - whether just once, a few times or as a regular partners, or as married partners.

6.1 Have you ever had any experiences of a sexual kind? E.g. of the various types described above. These may be brief contacts or longer term relationships.

Yes ☐ 1 No ☐ 2

If yes, please answer the remaining questions.
If no, turn to page 20.

6.2 If yes, when was the first time you ever had a relationship that involved :

	Age (or "never")	Was this since coming to University?
(a) Kissing and cuddling but not genital contact or intercourse		Yes ☐1 No ☐2
(b) Genital contact not involving intercourse		Yes ☐1 No ☐2
(c) Oral sexual intercourse		Yes ☐1 No ☐2
(d) Penetrative sexual intercourse		Yes ☐1 No ☐2

6.3 We would like to ask you about partners/contacts you have had, however brief, since coming to University (including during holidays).

Was the contact?	**Yes**	**No**	**How many males?**	**How many females?**
(a) Oral sexual intercourse **and** penetrative sexual intercourse	☐1	☐2	☐	☐
(b) Penetrative sexual intercourse not included in (a)	☐1	☐2	☐	☐
(c) Oral sexual intercourse not included in (a) or (b)	☐1	☐2	☐	☐
(d) Genital contact not involving intercourse not included in (a), (b) or (c)	☐1	☐2	☐	☐
(e) Kissing and cuddling but not leading to genital contact or intercourse not included in (a), (b), (c) or (d)	☐1	☐2	☐	☐

If you had penetrative sexual intercourse, did you use:

	Always or almost always	**Occasionally**	**Never or almost never**
A male condom:	☐	☐	☐
A female condom:	☐	☐	☐
A cap/diaphragm:	☐	☐	☐

6.4 Have you had a sexually transmitted disease since coming to University?

Yes ☐ 1 No ☐ 2

If yes, which of the following have you had?:	**Tick:** Yes	No
Gonorrhoea	☐ 1	☐ 2
Syphilis	☐ 1	☐ 2
NSU (chlamydia)	☐ 1	☐ 2
HIV	☐ 1	☐ 2
Genital warts	☐ 1	☐ 2
Genital herpes	☐ 1	☐ 2
Trichomonas	☐ 1	☐ 2

Other, please specify.....

THANK YOU VERY MUCH FOR HELPING US WITH THIS SURVEY

We appreciate your help, and hope that you have felt able to answer the questions without too much trouble. If there is anything you would like to add or comment upon, please write it below.

Visit Number	Number MRC/	Date of Visit
Date of Birth	Doctor	

Clinical assessment - Visit 1

Date of first symptoms (incl "prodrome")		
Date of most severe symptoms		
Duration of most severe symptoms (days)		
Unwell for how long (days)		
Presenting features and how long experienced for at time of presentation to GP (choice of physical features below)	1	
	2	
	3	

Activities

		Date stopped	Date Returned
Are you able to attend all your timetabled classes	Yes/No		
Total hours timetabled per week			
Please quantify since onset of symptoms: Exams missed Tutorials Lectures Practicals Total hours missed due to illness			
Is the amount of studying that you do in your own time normal for you at present?	Yes/No		
Are you able to undertake your normal social life?	Yes/No		
Are you able to undertake your normal exercise pattern ?	Yes/No		
Do you have a part-time job?	Yes/No		
Hours per week			
If yes, give dates absent, or "none" for no time off			

Physical features

Sore throat	Have you experienced this ever during your illness	No, not at any point during illness
Unable to swallow saliva	YES/NO	
Unable to swallow liquids	YES/NO	
Unable to swallow soft diet	YES/NO	
Able to swallow but painful	YES/NO	
Sore throat	YES/NO	
Rash a) faint morbilliform b) antibiotic related, (which and timing) c) other	YES/NO	
Glands	YES/NO	
Fever	YES/NO	
Sweats	YES/NO	
Anorexia	YES/NO	
Headaches	YES/NO	
Muscle/joint aches	YES/NO	
Syncope Abdominal pain Other		NO
Do you have any other medical conditions, the glandular fever has affected?		
Alcohol - units in last week		
Alcohol – usual per week		

Admitted to hospital / seen in A+E / out-patients?

Visit Number	**Number MRC/**	**Date of Visit**

Examination

Pulse	
Blood pressure	

Temperature	*student will be given chart and thermometer to do daily checks, data recorded separately*			
Throat exudate	*YES/NO*			
Periorbital swelling	YES/NO			
Palatial petechiae	YES/NO			
Jaundice	YES/NO			
Rash	YES/NO			
Spleen size	Not palpable	Tip	Fingers below costal margin	
Liver	Not palpable	Edge	Fingers below costal margin	
Palpable glands	YES/NO	cervical	Right left both	
		supraclavicular	Right left both	
		Submandibular	Right left both	
		axilliary	Right left both	
		inguinal	Right left both	
Tonsils enlarged	YES /NO	Grade 1 2 3		

Visit Number	**Number MRC/**	**Date of Visit**
Date of Birth	**Doctor**	

Visit Number_____ **Number MRC/** _____ **Date of Visit** _____

Date of Birth _____ **Doctor** _____

PHYSICAL FUNCTION AND FATIGUE

The following items are about activities you might do during a typical day and how your health now limits you in these activities.

1. Please rate your fatigue (weariness, tiredness) by circling the number that best describes your fatigue in the last week:

0	1	2	3	4	5	6	7	8	9	10
No fatigue										*As bad as you can imagine*

When your illness was at it's worst:

0	1	2	3	4	5	6	7	8	9	10
No fatigue										*As bad as you can imagine*

And before this current illness:

0	1	2	3	4	5	6	7	8	9	10
No fatigue										*As bad as you can imagine*

2. Record how many hours you spent on average per 24 hours in the last week:

(a) lying down but not asleep _____ hrs

(b) and asleep _____ hrs

3. *We want to assess how much your illness has affected your physical function*:

(a) Could you wash and dress at worst point in illness? YES/NO
Can you wash and dress now? YES/NO

(b) Could you leave your home at worst point in illness? YES/NO
Are you well enough to leave your home? YES/NO

(c) If you were able to leave home, please record how far you could walk easily e.g. a walk to the shops or college. (An average walking speed is 1km in 15 minutes)

At worst point in illness	Less than 100 metres	100-500 metres	500m-1km	1-2km	Over 2km
In the last week	Less than 100 metres	100-500 metres	500m-1km	1-2km	Over 2km
When well	Less than 100 metres	100-500 metres	500m-1km	1-2km	Over 2km

Visit Number_____ **Number MRC/** _____ **Date of Visit** _____

Date of Birth _____ **Doctor** _____

4. Please record how many hours per week you spend doing vigorous activity (enough to get out of breath) e.g. running, cycling, exercise class, swimming
at worst point in illness_____ **in last week**_____ **and when well**_____

5. Please record the number of hours per week you spent attending university sessions e.g. tutorials/lectures/ practical work
at worst point in illness_____ **in last week**_____ **and when well**_____

6. Please record hours per week you spent in private study (not including time recorded in question 5)
at worst point in illness_____ **in last week**_____ **and when well**_____

7. Has your illness affected your ability to concentrate? YES ☐ NO ☐

If yes please complete parts a-b, if no proceed to question ***8***

(a) Circle the one number that describes best how your illness has interfered with your ability to concentrate (e.g. keeping attentive over the course of a lecture or reading)

At the worst point in your illness:

0	1	2	3	4	5	6	7	8	9	10
No difficulty										*Unable to concentrate*

During the last week:

0	1	2	3	4	5	6	7	8	9	10
No difficulty										*Unable to concentrate*

Circle the one number that describes your ability to concentrate before you became unwell:

0	1	2	3	4	5	6	7	8	9	10
No difficulty										*Unable to concentrate*

(b) Again imagine you are in a lecture or reading a textbook: How long can you concentrate and learn new information for?

At the worst point in your illness? (circle)

5 minutes	10 minutes	20 minutes	40 minutes	Over 1 hour

Now?

5 minutes	10 minutes	20 minutes	40 minutes	Over 1 hour

8. Please record how many hours per week you spent going out to social events (such as seeing a film, going out to a pub, club, or out for a meal)
at worst point in illness_____ **in last week**_____ **and when well**_____

Clinical assessment- visits 2, 3 etc

Date of visit	Date
MRC Number	Visit number

Do you feel you are still experiencing your worst symptoms	YES/NO
If no when were you most unwell (in days after first became unwell)	Amount in days

Activities

		Date Returned
Are you able to attend all your timetabled classes	Yes/No	
Please quantify since last visit : Exams missed Tutorials Lectures Practicals Total hours		
Has the amount of studying that you do in your own time returned to normal?	Yes/No	
Has your social life returned to normal	Yes/No	
Has your exercise pattern returned to normal	Yes/No	
Do you have a part-time job	Yes/No	
If yes have you returned? (Date)		

Physical features

Sore throat	Have you experienced this since your last visit	No not since last seen
Unable to swallow saliva	YES/NO	
Able to swallow liquids	YES/NO	
Able to swallow soft diet	YES/NO	
Able to swallow but painful	YES/NO	
Throat not sore	YES/NO	
Rash a) faint morbilliform b) antibiotic related, (which and timing) c) other	YES/NO	
Glands	YES/NO	
Fever	YES/NO	
Sweats	YES/NO	
Anorexia	YES/NO	
Headaches	YES/NO	
Muscle/joint aches	YES/NO	
Anything else		NO
Do you have any other medical conditions, the glandular fever has affected?		
Alcohol- units in last week		

Examination

Pulse	
Blood pressure	

Temperature	*student will be given chart and thermometer to do daily checks, data recorded separately*			
Throat exudate	*YES/NO*			
Periorbital swelling	YES/NO			
Palatial petechiae	YES/NO			
Jaundice	YES/NO			
Rash	YES/NO			
Spleen size	Not palpable	Tip	Fingers below costal margin	
Liver	Not palpable	Edge	Fingers below costal margin	
Palpable glands	YES/NO	cervical	Right left both	
		supraclavicular	Right left both	
		submandibular	Right left both	
		axilliary	Right left both	
		inguinal	Right left both	
Tonsils enlarged	YES /NO	Grade 1 2 3		

Visit Number	**Number MRC/**	**Date of Visit**
Date of Birth	**Doctor**	

Visit Number_____ **Number MRC/** _____ **Date of Visit** _____

Date of Birth _____ **Doctor** _____

PHYSICAL FUNCTION AND FATIGUE

The following items are about activities you might do during a typical day and how your health now limits you in these activities.

1. Please rate your fatigue (weariness, tiredness) by circling the number that best describes your fatigue over the last week?

0	1	2	3	4	5	6	7	8	9	10
No fatigue										*As bad as you can imagine*

2. Record how many hours you spent on average per 24 hours in the last week:
(a) lying down but not asleep _____ hrs
(b) and asleep _____ hrs

3. *We want to assess how much your illness has affected your physical function*:

(a) Can you wash and dress now? YES/NO

(b) Are you well enough to leave your home? YES/NO

(c) If you are able to leave home, please circle how far you could walk easily e.g. a walk to the shops or college. (An average walking speed is 1km in 15 mins)

Less than 100m	100-500m	500m to 1km	1-2km	Over 2km

4. Please record how many hours per week you spend doing vigorous activity (Enough to get out of breath - e.g. running, cycling, exercise class)
in the last week _____ hrs

5. Please record the number of hours per week you spent attending university sessions e.g. tutorials/lectures/ practical work
in the last week _____ hrs

6. Please record hours per week you spent in private study (not including time recorded in question 5)
in the last week _____ hrs

Visit Number_____ **Number MRC/** _____ **Date of Visit** _____

Date of Birth _____ **Doctor** _____

7. Has your illness affected your ability to concentrate? YES ☐ NO ☐

If yes please complete parts a-b, if no proceed to question 8

(a) Circle the one number that describes best how your illness has interfered with your ability to concentrate (e.g. keeping attentive over the course of a lecture or reading)

During the last week:

0	1	2	3	4	5	6	7	8	9	10
No difficulty										*Unable to concentrate*

(b) Again imagine you are in a lecture or reading a textbook: How long can you concentrate and learn new information for?

In the last week:

5 minutes	10 minutes	20 minutes	40 minutes	Over 1 hour

8. Please record how many hours per week you spent going out to social events (such as seeing a film, going out to a pub, club, or out for a meal)

in the last week_____ hrs

EDINBURGH STUDENT QUESTIONNAIRE

GLANDULAR FEVER FORM

CONFIDENTIAL

ID Number:	Male ☐ Female ☐		
Date of Birth	☐☐ Day	☐☐ Month	☐☐ Year
Date of Interview	☐☐ Day	☐☐ Month	☐☐ Year

Introduction

You may recall that at the beginning of your course you filled in a questionnaire and gave a blood sample as part of a study investigating the ways in which your lifestyle and background relate to your risk of developing glandular fever. Now that you have glandular fever, we would appreciate it if you would fill in a further questionnaire and allow us to take some more blood. Once again your replies will be treated confidentially. The information will not be disclosed to the University or others. Your participation is voluntary and whether you participate or not will make no difference to your medical care whilst at the University. If there are some questions that you would prefer not to answer, that is fine – just leave a blank. If you wish to discuss any aspect of the study further please contact Dr Tanzina Haque*, Lecturer in Virology, who is not directly involved in the study.

Thank you, in anticipation, for your help.

Dorothy H Crawford
Professor of Medical Microbiology
University of Edinburgh

Dr Nadine Harrison
University Health Service
University of Edinburgh

* Dr. Tanzina Haque, Lecturer in Virology, Tower Block, Summerhall, University of Edinburgh (0131-650-7941).

1. *QUESTIONS ABOUT YOUR STUDIES*

1.1 When did you start at Edinburgh University?

.....

1.2 What course are you undertaking?

Please specify .....

1.3 In the past 6 months have you:

	Yes	No
Changed course	☐	☐
Taken exams	☐	☐
Undertaken work contributing to your final degree mark	☐	☐

2. *PERSONAL AND FAMILY DETAILS*

Prior to attending primary school, what were the arrangements for looking after you, and for approximately how many years did these apply? Tick more than one box if appropriate

	Yes	Age started (years)	No	Don't know
Nursery school or reception class	☐	☐	☐	☐
Other day care centre	☐	☐	☐	☐
At home with mother or carer	☐	☐	☐	☐
At childminder's home	☐	☐	☐	☐
Other outside home – please specify	☐	☐	.....	

2.1 Are you:

	tick:
Married?	☐ 1
Widowed?	☐ 2
Divorced or Separated?	☐ 3
Cohabiting (living with partner)?	☐ 4
In a relationship, but not living with partner?	☐ 5
Single?	☐ 6

Has this changed in the past 6 months? Yes ☐ 1 No ☐ 2

2.2 Do you have any children? Yes ☐1 No ☐2 **If yes:**

Sex:		**Date of Birth:**		
Male ☐1	Female ☐2	☐☐ Day	☐☐ Month	☐☐ Year
Male ☐1	Female ☐2	☐☐ Day	☐☐ Month	☐☐ Year
Male ☐1	Female ☐2	☐☐ Day	☐☐ Month	☐☐ Year
Male ☐1	Female ☐2	☐☐ Day	☐☐ Month	☐☐ Year

3. *QUESTIONS ABOUT YOUR LIVING CIRCUMSTANCES*

3.1 Please list each residence you have lived in for 1 month or more during the past 6 months:

1ST RESIDENCE:

(a) from ☐☐ / ☐☐ (M / Y) to ☐☐ / ☐☐ (M / Y)

Village/town/city ..... Country .....

Type of accommodation: please tick:

Halls of residence	☐
House	☐
Flat	☐
Other	☐ please specify .....

With how many adults and how many children on average did you share the following rooms?:

	Adults	Children
Kitchen	☐	☐
Bathroom	☐	☐
Bedroom	☐	☐
Living room	☐	☐

3.2 **2nd RESIDENCE:**

(b) from ☐☐ / ☐☐ (M / Y) to ☐☐ / ☐☐ (M / Y)

Village/town/city ..... Country .....

Type of accommodation: **please tick:**

Halls of residence	☐
House	☐
Flat	☐
Other	☐ please specify .....

With how many adults and how many children on average did you share the following rooms?:

	Adults	Children
Kitchen	☐	☐
Bathroom	☐	☐
Bedroom	☐	☐
Living room	☐	☐

3.3 **3rd RESIDENCE:**

(c) from ☐☐ / ☐☐ (M / Y) to ☐☐ / ☐☐ (M / Y)

Village/town/city ..... Country .....

Type of accommodation: **please tick:**

Halls of residence	☐
House	☐
Flat	☐
Other	☐ please specify .....

With how many adults and how many children on average did you share the following rooms?:

	Adults	Children
Kitchen	☐	☐
Bathroom	☐	☐
Bedroom	☐	☐
Living room	☐	☐

If you require more space please continue at the end of the questionnaire.

4. *MEDICAL BACKGROUND*

4.1 During the past 6 months have you been immunised against?:

	please tick: Yes	No	Don't know
Polio	☐ 1	☐ 2	☐ 8
Rubella	☐ 1	☐ 2	☐ 8
TB (BCG)	☐ 1	☐ 2	☐ 8
Typhoid	☐ 1	☐ 2	☐ 8
Tetanus	☐ 1	☐ 2	☐ 8
Hepatitis A	☐ 1	☐ 2	☐ 8
Hepatitis B	☐ 1	☐ 2	☐ 8
Meningitis	☐ 1	☐ 2	☐ 8
Yellow Fever	☐ 1	☐ 2	☐ 8
Japanese B Encephalitis	☐ 1	☐ 2	☐ 8
Rabies	☐ 1	☐ 2	☐ 8
Diphtheria	☐ 1	☐ 2	☐ 8
Other	☐ 1	☐ 2	☐ 8

Other, please state.....

4.2 Have you had any illnesses during the past 6 months, apart from your present glandular fever that required a visit to your doctor?

Yes ☐ No ☐

If yes, was the visit for:	**Tick:** Yes	**Tick:** No	**Tick:** Time off study (days)
Short illness with full recovery (eg 'flu) Please specify	☐ 1	☐ 2	☐
On-going medical condition (eg diabetes) Please specify	☐ 1	☐ 2	☐
Other illness Please specify	☐ 1	☐ 2	☐

4.3 Have you experienced psychological/mental illness during the past 6 months. If yes, was this:

	Tick: Yes	Tick: No	Did you receive medical treatment? Yes	Did you receive medical treatment? No
Depression	☐ 1	☐ 2	☐ 1	☐ 2
Drug overdose	☐ 1	☐ 2	☐ 1	☐ 2
Anorexia	☐ 1	☐ 2	☐ 1	☐ 2
Bulimia	☐ 1	☐ 2	☐ 1	☐ 2
Schizophrenia	☐ 1	☐ 2	☐ 1	☐ 2
Drug/alcohol related problems	☐ 1	☐ 2	☐ 1	☐ 2
Stress-related problems	☐ 1	☐ 2	☐ 1	☐ 2

Other, please state .....

4.4 Have you experienced any of the following stressful events during the past 6 months?

	Tick: Yes	Tick: No	If Yes, When?
Your parent, child, sibling or partner was seriously ill or died?	☐ 1	☐ 2	
You broke off a marriage or steady relationship?	☐ 1	☐ 2	
You had a major financial crisis?	☐ 1	☐ 2	
Financial pressure led to you taking a part-time job?	☐ 1	☐ 2	
Major problems with your studies/exams?	☐ 1	☐ 2	

Other, please state .....

4.5 Have you used any medication (including oral contraceptives and inhalers) during the three months before you got glandular fever?

Yes ☐ 1 No ☐ 2 **If yes, please state which and when:**

1.
2.
3.
4.
5.
6.

(please continue at end of questionnaire if necessary)

4.6 (a) Do you know anyone who has had glandular fever in the last 3 months?

Yes ☐ 1 No ☐ 2 If yes, how many? ☐☐

(If you have had contact with more than one person please record the first contact here and the second in the next section below)

What was your relationship to the person?
Please specify.....

(b) Do/did you live with this person whilst they had glandular fever or during the 3 months preceding their illness?

Yes ☐ 1 No ☐ 2

Was it in a:

House	Yes ☐ 1	No ☐ 2
Flat	Yes ☐ 1	No ☐ 2
Hall of residence	Yes ☐ 1	No ☐ 2

Did you share a:

Bedroom	Yes ☐ 1	No ☐ 2
Kitchen	Yes ☐ 1	No ☐ 2
Bathroom	Yes ☐ 1	No ☐ 2

(c) How often do/did you see them whilst they had glandular fever? Was it:

Daily	Yes ☐ 1	No ☐ 2
At least Weekly	Yes ☐ 1	No ☐ 2
Less than weekly	Yes ☐ 1	No ☐ 2

Please complete this if you have had contact with a second person who has had glandular fever in the last 3 months

4.6 Second contact

What was your relationship to the person?

Please specify.....

(c) Do/did you live with this person whilst they had glandular fever or during the 3 months preceding their illness?

Yes ☐1 No ☐2

Was it in a:

House	Yes ☐1	No ☐2
Flat	Yes ☐1	No ☐2
Hall of residence	Yes ☐1	No ☐2

Did you share a:

Bedroom	Yes ☐1	No ☐2
Kitchen	Yes ☐1	No ☐2
Bathroom	Yes ☐1	No ☐2

(d) How often do/did you see them whilst they had glandular fever? Was it:

Daily	Yes ☐1	No ☐2
At least Weekly	Yes ☐1	No ☐2
Less than weekly	Yes ☐1	No ☐2

4.7 Have you had your tonsils out? Yes ☐1 No ☐2

If so, at what age? .....

5. *SMOKING, DRINKING, EXERCISE AND TRAVEL*

5.1 Smoking

Have you smoked tobacco (ie smoked at least 1 cigarette or cigar or pipe per day, on average) during the last 6 months? Yes ☐1 No ☐2

If yes, how, much, on average, did you smoke before you got glandular fever?

cigarettes / day ☐☐

cigars / day ☐☐

hand rolling tobacco oz / week ☐☐

pipe tobacco oz / week ☐☐

5.2 Alcohol

Had you drunk alcohol during the past 6 months before you got glandular fever?

Yes ☐1 No ☐2

If yes, how much, on average, did you drink before your present illness?

Beer or cider (pints) ☐☐ per day **or** ☐☐ per week

Wine (glasses) ☐☐ per day **or** ☐☐ per week

Spirits (tots) ☐☐ per day **or** ☐☐ per week

5.3 Exercise

During the past 6 months (but before your present illness) have you regularly (once a week or more) engaged in active sports or other active exercise sufficient to raise a sweat or get you out of breath for at least 20 minutes ?

Yes ☐1 No ☐2

If yes, number of hours / week on average: ☐☐

5.4 Travel

Have you travelled outside the UK in the past 6 months? Yes ☐1 No ☐2

1ST VISIT

Which country? (name) .....

When did you go to the country?.....

How long was the visit? .....

Did you suffer from travel-related illness? Yes ☐1 No ☐2

If yes, please specify .....
.....

2ND VISIT

Which country? (name) .....

When was the visit? .....

How long was the visit? .....

Did you suffer from travel-related illness? Yes ☐1 No ☐2

If yes, please specify .....
.....

3RD VISIT

Which country? (name) .....

When was the visit? .....

How long was the visit? .....

Did you suffer from travel-related illness? Yes ☐1 No ☐2

If yes, please specify .....
.....

If you have visited more countries, please continue at end.

6. *SEXUAL RELATIONSHIPS*

Glandular fever is passed on by close contact, and so it is useful for us to know about your sexual relationships.

Confidentiality:
We are aware that the questions in this section are personal. Your answers will be treated in strict confidence; the doctor will not see them. When you have finished put the questionnaire in the envelope, your name will not be on the questionnaire or envelope.

If you do not feel happy about answering these questions that is fine, just leave them out. We have given you some notes, to ensure everyone applies the same meaning to certain terms we use. Please read the appendix before continuing.

6.1 Have you ever had any experiences of a sexual kind? E.g. of the various types described in the appendix . These may be brief contacts or longer term relationships.

Yes ☐ 1 No ☐ 2

If yes, please answer the remaining questions.

6.2 If yes, when was the first time you ever had a relationship that involved :

	Age (or "never")	Was this in the last three months
(a) Kissing and cuddling but not genital contact or intercourse		Yes ☐ 1 No ☐ 2
(b) Genital contact not involving intercourse		Yes ☐ 1 No ☐ 2
(c) Oral sexual intercourse		Yes ☐ 1 No ☐ 2
(d) Penetrative sexual intercourse		Yes ☐ 1 No ☐ 2

6.2. Have you had any sexual relationships in the last 3 months before you developed glandular fever? If yes, we would like to ask you some questions about the relationship(s), however brief:

Number of relationships in past 3 months? ☐☐

Number of Males? ☐

Number of Females? ☐

If you have had 1 or 2 relationships please in the last 3 months complete section 6.2 only. If there were more than 2, please complete sections 6.2 **and** 6.3.

RELATIONSHIP 1

What sex was the person? Male ☐ Female ☐

Was the contact?	**Yes**	**No**	**If yes on how many occasions in the last three months did this occur?**
(a) Kissing and cuddling but not leading to genital contact or intercourse	☐1	☐2	.....
(b) Genital contact not involving intercourse	☐1	☐2	.....
(c) Oral sexual intercourse	☐1	☐2	.....
(d) Penetrative sexual intercourse.	☐1	☐2	.....
(e) Oral sexual intercourse and penetrative sexual intercourse	☐1	☐2	.....

If you had penetrative sexual intercourse, did you use:

	Always	**Occasionally**	**Never**
A male condom:	☐	☐	☐
A female condom:	☐	☐	☐
A cap/diaphragm:	☐	☐	☐

Contact Code

RELATIONSHIP 2

What sex was the person? Male ☐ Female ☐

Was the contact?	**Yes**	**No**	**If yes on how many occasions in the last three months did this occur?**
(b) Kissing and cuddling but not leading to genital contact or intercourse	☐1	☐2	.....
(b) Genital contact not involving intercourse	☐1	☐2	.....
(c) Oral sexual intercourse	☐1	☐2	.....
(f) Penetrative sexual intercourse.	☐1	☐2	.....
(g) Oral sexual intercourse and penetrative sexual intercourse	☐1	☐2	.....

If you had penetrative sexual intercourse, did you use:

	Always	**Occasionally**	**Never**
A male condom:	☐	☐	☐
A female condom:	☐	☐	☐
A cap/diaphragm:	☐	☐	☐

Contact Code

6.3 Have you had any other relationships however brief in the last 3 months before your illness? If yes, we would like to ask you some questions about these relationships.

Was the contact?	Yes	No	If yes, with how many people in the last three months did this occur? *(not including relationship 1 & 2)* How many males?	How many females?
(a) Kissing and cuddling but not leading to genital contact or intercourse	☐1	☐2	☐	☐
(b) Genital contact not involving intercourse	☐1	☐2	☐	☐
(c) Oral sexual intercourse	☐1	☐2	☐	☐
(d) Penetrative sexual intercourse.	☐1	☐2	☐	☐
(e) Oral sexual intercourse and penetrative sexual intercourse	☐1	☐2	☐	☐

If you had penetrative sexual intercourse, did you use:

	Always	Occasionally	Never
A male condom:	☐	☐	☐
A female condom:	☐	☐	☐
A cap/diaphragm:	☐	☐	☐

6.4 Have you had a sexually transmitted disease during the past 6 months?

Yes ☐1 No ☐2

If yes, which of the following have you had?:

	Tick: Yes	No
Gonorrhoea	☐1	☐2
Syphilis	☐1	☐2
NSU (chlamydia)	☐1	☐2
HIV	☐1	☐2
Genital warts	☐1	☐2
Genital herpes	☐1	☐2
Trichomonas	☐1	☐2

Other, please specify.....

THANK YOU VERY MUCH FOR HELPING US WITH THIS SURVEY

We appreciate your help, and hope that you have felt able to answer the questions without too much trouble. If there is anything you would like to add or comment upon, please write it below:-

_____

_____

_____

_____

APPENDIX

Please read these notes before answering the questions:

They are just to make sure everyone applies the same meaning to certain terms we have used.

Genital area
A man's penis or woman's vagina- that is sex organs.

(a) **Any sexual contact or experience**
That is a wider term and can include just kissing or cuddling, but not leading to genital contact or intercourse

(b) **Genital contact NOT involving intercourse**
Forms of contact with the genital area NOT leading to intercourse (vaginal, oral or anal) but intended to be sexually stimulating, for example, stimulating by hand.

(c) **Oral sexual intercourse**
A man's or woman's mouth on a partner's genital area

(d) **Penetrative sexual intercourse**
Either vaginal or anal sexual intercourse

Partner/relationship
People who have had sexual experiences together, of any type (a), (b), (c) and (d) referred to above - whether just once, a few times or as a regular partners, or as married partners.

12.2 EBV Type and Lymphocyte Count at Diagnosis

The number of IM cases caused by type 2 strains was insufficient to permit investigation of any associations between EBV type and clinical symptoms, laboratory parameters or viral load. Lymphocyte counts and EBV type were made available to me for an additional ten IM cases by Drs. R. Thomas and K. McAulay. Interestingly IM caused by type 1 may be associated with a higher lymphocyte count in the peripheral blood

EBV type	n	Median lymphocyte count at diagnosis $x10^9 l^{-1}$	p value
1	44	5.77	-
2	4	2.86	p=0.022
Both types	4	6.03	0.837

n Number of students

Includes data from an additional 10 IM cases supplied by Drs. Thomas and McAulay.

Table 25 EBV type and Lymphocyte count at Diagnosis

12.3 Gender Composition of University Students

The gender composition of the student body eligible to enter the study was estimated from the following information kindly made available by Andrew Quickfall in The Governance and Strategic Planning Department of University of Edinburgh. In 1999-2000 there were 3851 undergraduates of whom 54% were female, and 596 PhD students of whom 47% were female. In the year 2000-2001 there were 3988 undergraduates of whom 54% were female and 626 PhD students of whom 46% were female. Overall this means that 53% of eligible students in the university as a whole were female in these two years.

12.4 Comparison of Lymphocyte Subset Ranges for Controls with Published Data

The results of lymphocyte subset percentages obtained for the healthy control group in this study are in line with other published data as shown in Table 26. The results are presented as average (mean) parentages as this allows comparison with the published data. Actual lymphocyte numbers rather than percentages were used in the current study as the use of percentages to describe lymphocyte changes may be misleading, and this is particularly true in IM where the acute lymphocytosis does not affect the lymphocyte subsets equally.

	Reichert *et al* (1991) 18-70 years (4 centres) n=261	Howard *et al* (1996) 18-67 years (Caucasian) n=79	Bofill *et al* (1992) 11-79 years n=600	Williams *et al* (1983) 21-53 years n=20	Mean of control group Edinburgh n=15
CD3%	69.9-74.1	72.9	76.3	74.8	71.6
CD8%	31.4-36.1	24.7	29.5	27.4	29.2
CD4%	42.9-45.0	46.8	43.6	52.5	45.0
CD19%	12.8-14.6	-	-	-	17.7
CD56%	12.9-16.9	-	-	-	10.7
CD4%/CD8%	1.28-1.56	2.1	1.50	1.92 (calc)	1.67

calc calculated

Table 26 Comparison of lymphocyte subset ranges in controls with other published data

Reichert *et al* compared results from healthy adults aged 18 to 70 years at four centres (Reichert *et al*, 1991). Data from persons aged 18 to 30 years made up 24% of the samples, but unfortunately the results were not presented by age. A number of age related changes in lymphocyte subset percentages have been described including a 1% rise in the percentages of NK and $CD4^+$ cells per decade and a 1% fall in $CD8^+$ percentage per decade (Bofill *et al*, 1992; Reichert *et al*, 1991). This illustrates the importance of the use of age-matched controls in this study. The rise in NK percentage with age may explain the lower NK percentage in controls in the current study (10.7%), where all participants were less than 30 years of age, than in the study by Reichert *et al* (1991) who reported a range of 12.9 to 16.9% NK cells. Howard *et al*. compared data from Asians and other ethnic groups. Their results from Caucasians are shown in Table 26 above, together with data from Reichert *et al*, a study performed at The Royal Free Hospital, UK, and, a small study from New Mexico (Bofill *et al*, 1992; Howard *et al*, 1996; Reichert *et al*, 1991; Williams *et al*, 1983). In health the number of $CD4^+$ T-cells is greater than that of the $CD8^+$ T-cells such that the ratio of CD4:CD8 T-cells is at least 1.0. Study of control students in Edinburgh University are in line with this finding, with an overall CD4:CD8 ratio for 15 controls of 1.57. Ten to 20 percent of normal controls have been noted to have CD4:CD8 ratios less than 1 but the magnitude of the shortfall was not documented (Bofill *et al*, 1992; Reichert *et al*, 1991). In the current study three controls had CD4:CD8 ratios of less than 1, although the actual

values were very close to 1 (0.97, 0.95 and 0.96). Overall, these comparisons show that the control data in this study is in line with other published data.

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