with the monoclonal antibody. As anticipated, some monoclonal antibodies were able to neutralise XMRV (83A25' and 609) whilst others had no effect on XMRV infectivity. Interestingly, we identified three monoclonal antibodies that neutralised MLV(X) but not XMRV (603, 610 and 613) and one that neutralised XMRV but not MLV(X) (609). These reagents may therefore be useful tools with which to distinguish XMRV from other xenotropic MLVs in future investigations. From these experiments we defined two negative (603 and 613) and one positive (83A25') antibody controls for further experiments. To validate the neutralisation assay and examine the possible range of responses to "normal serum", we tested neutralisation using a panel of 226 serum samples from BLT. Previous investigations have detected XMRV DNA in ~1-6% of control samples [5,6,8]. Of our panel only a handful showed possible neutralisation activity, giving curves similar to that shown in Figure 2A, with reductions in viral infectivity similar or greater than that seen with the positive control, monoclonal 83A25'. Over 90% of the samples tested had less than a 2-fold effect on infectivity (Figure 3A). From these data, we have defined a positive as a sample that reduces viral infectivity by at least 70% at a dilution of 1/40 and gives a reduction of 50% at a 1/80 dilution. According to this definition, the BLT sample set contains 3 neutralising sera, identifying 1.3% of samples as positive.

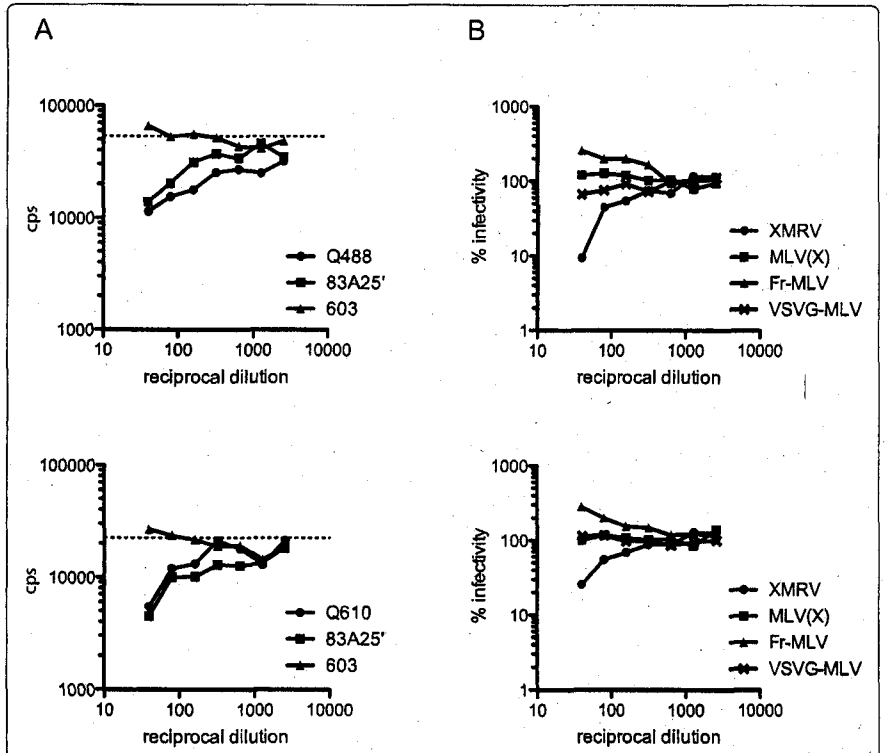

Figure 2 Examples of BLT positive serum neutralisation activity. A, Infectivity of XMRV (measured as counts per second of chemiluminescence produced from β-galactosidase activity) after incubation with patient serum or hybridoma cell supernatant. Infectivity is plotted against the reciprocal dilution of the BLT serum (black circles, top panel, sample Q488, bottom panel, sample Q610; triangles, negative control, monoclonal 603; squares, positive control, monoclonal 83A25'). The dashed line indicates viral infectivity in the absence of sera. B, Infectivity data for viruses with four different envelopes (circles, XMRV; squares, MLV(X); triangles, Friend-MLV; crosses, VSV-G) after incubation with patient serum. Data were normalised by setting the infectivity for each virus in the absence of patient serum at 100%, and plotted against the reciprocal of serum dilution for two positive sera, top panel sample Q488 and bottom panel sample Q610.

To confirm that the neutralisation activity demonstrated was specific for XMRV, we tested a subset of sera for neutralisation of XMRV alongside MLV particles pseudotyped with different envelope proteins from MLV (X), Friend-MLV or VSV. As shown in Figure 2B, of these four virus preparations, only XMRV infectivity was inhibited by any of the sera tested. Even the infectivity of particles expressing the closely related MLV(X) envelope that is 94% identical to XMRV was unaffected by sera that inhibited XMRV (Figure 2B, squares). Thus, it seems that the neutralising activity is specific for XMRV.

We therefore felt this assay was sensitive and specific enough to examine the neutralising ability of the SGUL cohort of blinded patient serum samples. After unblinding the samples, it emerged that of the 142 CFS patient sera tested none was positive as defined by the criteria above (Figure 3B). These results suggested that there was no link between XMRV and CFS. By contrast, the control group of 157 blood donors contained 22 positives, a frequency of 14%, considerably higher than that seen in the BLT group (Figure 3C). It was also noticeable that the neutralising activity of all but one of the SGUL positive samples was much stronger than the BLT positive samples (compare Figure 2A with Figure 4A). In fact, most of the SGUL positive sera reduced XMRV infectivity by 100 fold at both 1/40 and 1/80 dilutions. Intriguingly, many of these serum samples were collected from a single blood donation session. Some samples from this session, however, were negative. Surprisingly, PCR analyses of DNA samples corresponding to the positive sera from the SGUL controls were uniformly negative. We therefore investigated the specificity of this response by testing 21 of the positive sera for neutralisation of MLV pseudotyped with the envelope proteins from MLV(X), Friend-MLV or VSV. In every case, the serum was able to neutralise additional viruses to XMRV, including particles pseudotyped with the non-retroviral envelope from VSV (Figure 4B and Table 3). This implied that the strong positive neutralising activity demonstrated by the SGUL blood donor controls was not specific to XMRV, and in all likeliness was not elicited by this virus.

To test whether the SGUL cohort of CFS patients was unique, we also tested 40 samples (including some

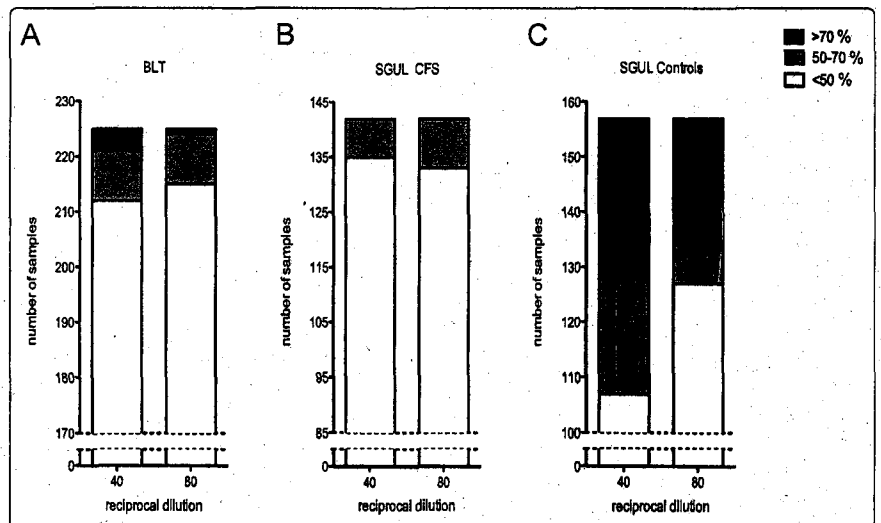

Figure 3 Distribution of neutralisation activity in three samples sets. Numbers of patients showing different degrees (>70% , 50-70% and <50%) of neutralisation of XMRV infectivity are shown for the 1/40 and 1/80 serum dilutions. A, Total BLT cohort (n = 226); B, SGUL CFS cohort (n = 142); C, SGUL control blood donor cohort (n = 157).

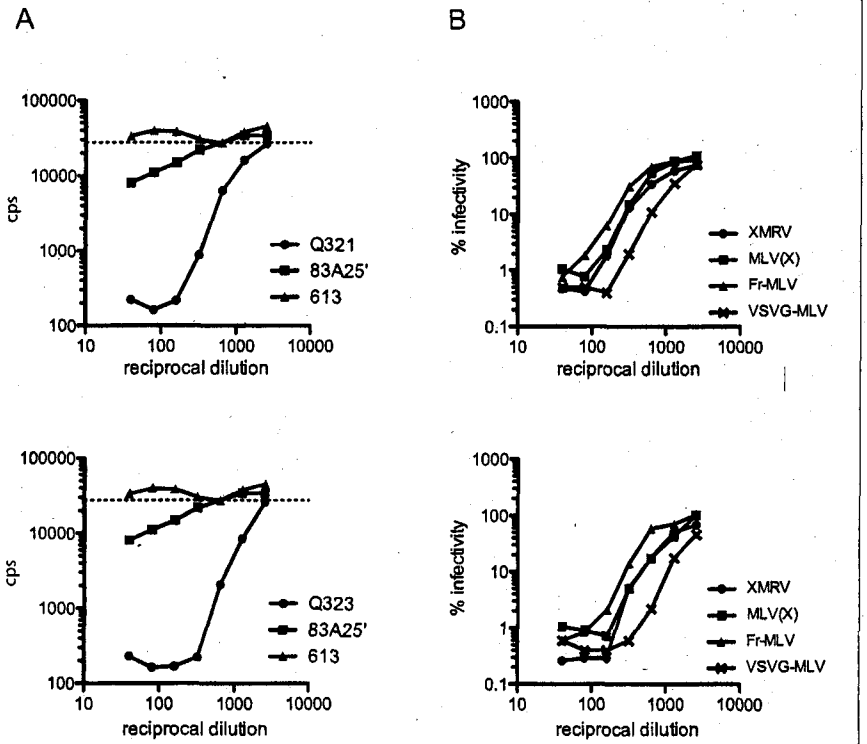

Figure 4 Examples of SGUL positive serum neutralisation activity. A, Infectivity of XMRV (measured as counts per second of chemiluminescence produced from β-galactosidase activity) after incubation with patient serum or hybridoma cell supernatant. Infectivity is plotted against the reciprocal dilution of the SGUL serum (black circles, top panel, sample Q321, bottom panel, sample Q323; triangles, negative control, monoclonal 613; squares, positive control, monoclonal 83A25'). The dashed line indicates viral infectivity in the absence of sera. B, Infectivity data for viruses with four different envelopes (circles, XMRV; squares, MLV(X); triangles, Friend-MLV; crosses, VSV-G) after incubation with patient serum. Data were normalised by setting the infectivity for each virus in the absence of patient serum at 100%, and plotted against the reciprocal of serum dilution for two positive sera, top panel sample Q321 and bottom panel sample Q323.

plasma samples as well as sera) from a separate CFS cohort in our neutralisation assay. This GC cohort revealed a solitary positive out of 28 CFS samples (3.6%), and no positives out of 12 control samples. The positive CFS patient serum was also able to neutralise MLV pseudotyped with either MLV(X) or Friend envelopes, although interestingly, it was not able to neutralise VSV-G pseudotyped MLV (Table 3). Neutralisation data from the different cohorts are summarized in Table 4. Thus, in summary, we found no association of XMRV with either CFS cohort.

Discussion

We set out with the intention of confirming the results of Lombardi *et al.* [8] concerning the association of XMRV with CFS. In total, we tested 142 CFS samples for both the presence of XMRV DNA in PBMCs by PCR and for the presence of neutralising antibodies against XMRV in our viral neutralisation assay, and a further 28 CFS samples for neutralising antibodies only. However, in contrast to Lombardi *et al.*, we found no evidence of XMRV DNA in any patient samples tested, and only a single neutralisation-positive patient serum. Our findings

Table 3 Neutralisation properties of different human sera against XMRV and MLV pseudotyped with three different envelopes.

	Neutralisation of				
Sample ID	XMRV	MLV(X)	Friend	VSV	XMRV detected by PCR
Barts and the London					
Q488	+	-	-	-	ND
Q610	+	-	-	-	ND
Q663	+	ND	ND	ND	ND
St George's University of London					
Q302	++	++	++	++	no
Q304	++	++	++	++	no
Q305	++	++	++	++	no
Q306	++	++	++	++	no
Q307	++	+	+	-	no
Q308	++	++	++	++	no
Q309	++	++	++	++	no
Q310	++	++	++	++	no
Q311	++	+	+	+	no
Q312	++	++	++	++	no
Q313	++	++	++	++	no
Q314	++	ND	ND	++	no
Q315	++	++	++	++	no
Q316	++	++	++	++	no
Q317	++	++	++	++	no
Q319	++	ND	ND	++	no
Q320	++	++	++	++	no
Q321	++	++	++	++	no
Q323	++	++	++	++	no
Q324	++	++	++	++	no
Q326	++	ND	ND	ND	no
Q372	+	-	-	+	no
Glasgow Calendonian University					
Q125	+	++	++	-	ND

+ indicates neutralising activity; ++ indicates strong neutralising activity; - indicates no neutralising activity; ND is no determined.

therefore appear inconsistent with the previous report that isolated XMRV from PBMCs of CFS patients. We are confident that, although we are unable to replicate the PCR detection of XMRV in PBMC DNA from CFS patients, our PCR assay is more sensitive than the published single round PCR method and should have possessed the necessary sensitivity to detect XMRV if it was indeed present (Figure 1). Furthermore, we were able to detect neutralising activity in one patient and in several control serum samples (Table 4 and Figure 3), implying that our neutralisation assay also has the required sensitivity. The lack of neutralising activity in CFS samples compared to controls could reflect an inability to mount an immune response in these patients. However, in that case, the virus would be expected to replicate to higher levels in CFS patients making it easier to detect by PCR. As we could not detect any evidence of XMRV infection by our PCR assays, we think this is an unlikely explanation. Thus, in our cohorts, we found no association of XMRV with CFS. This is in stark contrast to the result of Lombardi *et al.* [8]. However, it is thought likely that the term CFS defines multiple diseases [15-17], and it remains formally possible that a fraction of these are associated with XMRV. During the submission of this manuscript another report was published by Erlwein *et al.* that also failed to detect XMRV in CFS patients by PCR [18]. The publication of these results has promoted much discussion and controversy amongst CFS researchers and patients alike, and has highlighted the need for additional investigations in this area. Following the findings reported here, it would seem a prudent next step for subsequent studies to compare samples and protocols between different laboratories around the world.

There have also been conflicting reports describing the association of XMRV with prostate cancer. Two studies from the USA [1,5] have found an increased

Table 4 Summary of number of positive sera with XMRV neutralisation properties

Sample cohort	Positive	Total number
Barts and the London		
Control	3	226
St Georges University of London		
CFS	0	142
Control	22	157
Glasgow Caledonian University		
CFS	1	28
Control	0	12

prevalence of the virus in prostate cancer patients, although they differed as to whether this was dependent on the RNASEL genotype of the patient. Conversely, two German studies failed to establish a link between the virus and disease [6,7]. Nevertheless, XMRV has been detected in the control groups in multiple investigations [5,6,8], with the incidence varying between 1 and 6%. In our serological studies we have also identified neutralising activity against XMRV in around 4% of all the samples examined. Remarkably many (but not all) of the seropositive samples were identified in a relatively small group of blood donors within the SGUL cohort, possibly suggesting a local outbreak of infection. There is no evidence that this group are related or that they have a particularly high risk of acquiring a retroviral infection. Therefore, an outbreak of this kind seems unlikely. Moreover, all but one of the positive samples from the SGUL set we tested were also able to neutralise MLV pseudotyped with the envelope protein from VSV (Table 3). The one serum that failed to neutralise VSV-G pseudotyped MLV was, however, able to neutralise MLV particles pseudotyped with other retroviral envelopes. We therefore consider these positives from healthy blood donors to be non-specific cross reacting responses. The remaining four positive samples from the BLT and GC cohorts had much weaker neutralisation activities and did not neutralise VSV-G pseudotyped MLV, although, again, the positive serum from GC did neutralise particles expressing other retroviral envelopes (Table 3). Although we cannot rule out the possibility that the activity of these samples against XMRV is also non-specific, one possible explanation for these serological findings remains that XMRV infection has occurred in around one percent of the population. This figure is consistent with the general prevalence in control samples previously reported. Given the common oncogenic properties of gammaretroviruses [19] and the reported link between XMRV and prostate cancer [1,5], such an observation might be of considerable significance, particularly for the blood transfusion services. It should, however, be noted that we have so far been unable to reliably detect bacterially expressed XMRV Gag proteins by using these sera in immunoblotting experiments. It is therefore conceivable that these neutralising activities were not elicited by XMRV. Further investigations are required to determine the nature of these antiviral activities.

Conclusions

In summary, we have studied 299 DNA samples and 565 serum samples for evidence of XMRV infection. We have not identified XMRV DNA in any samples by PCR, however, some serum samples were able to neutralise XMRV infectivity in our assay. Only one of these positive sera came from a CFS patient, implying that there is no association between XMRV infection and CFS. Furthermore, most of the positive sera were also able to neutralise MLV particles pseudotyped with other envelope proteins, indicating there may be cross reactivity with other retroviruses and even other enveloped viruses. It therefore seems unlikely that these responses were elicited by XMRV. However, the detection of neutralising activity that did not neutralise VSV-G pseudotyped MLV in at least four human sera may indicate that XMRV infection does occur at in the general population, although the outcome of such infections is currently uncertain.

Acknowledgements

This work was supported by the UK Medical Research Council (file reference (KB) U117592729 and (JS) U117512710), The Wellcome Trust (grant ID 084955) and CFS Research Foundation, UK. We acknowledge The Cunningham Trust for funding to SH. KNB is a Wellcome Trust Career Development Fellow. We thank Leonard Evans for anti-MLV Env hybridoma supernatants and Robin Weiss and Nigel Temperton for helpful advice about neutralisation assays. We thank the following clinicians who provided patients for the present study; Dr Selwyn Richards, Dr Janice Main, Prof David J Nutt, Dr David Honeybourne, Dr Luis Nacul, Dr Amolak Bansal, Prof Peter Behan and Dr Abhijit Chaudhuri, and Mark Quinlivan for retrieving samples.

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Authors' contributions

JK, JS and KB conceived and designed the investigation. HG and VB carried out the viral neutralisation assays and analysed the data. KM, ER, SB and JK performed the PCR analyses. SH, JG, FM, JB and JK provided patient samples. JS and KB analysed the data and drafted the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Received: 11 January 2010
Accepted: 15 February 2010 Published: 15 February 2010

References

1. Urisman A, Molinaro RJ, Fischer N, Plummer SJ, Casey G, Klein EA, Malathi K, Magi-Galluzzi C, Tubbs RR, Ganem D, Silverman RH, DeRisi JL: **Identification of a novel Gammaretrovirus in prostate tumors of patients homozygous for R462Q RNASEL variant.** *PLoS Pathog* 2006, **2**:e25.
2. Dong B, Kim S, Hong S, Das Gupta J, Malathi K, Klein EA, Ganem D, Derisi JL, Chow SA, Silverman RH: **An infectious retrovirus susceptible to an IFN antiviral pathway from human prostate tumors.** *Proc Natl Acad Sci USA* 2007, **104**:1655-1660.
3. Jern P, Stoye JP, Coffin JM: **Role of APOBEC3 in genetic diversity among endogenous murine leukemia viruses.** *PLoS Genet* 2007, **3**:2014-2022.
4. Coffin JM, Stoye JP: **Virology. A new virus for old diseases?.** *Science* 2009, **326**:530-531.
5. Schlaberg R, Choe DJ, Brown KR, Thaker HM, Singh IR: **XMRV is present in malignant prostatic epithelium and is associated with prostate cancer, especially high-grade tumors.** *Proc Natl Acad Sci USA* 2009, **106**:16351-16356.
6. Fischer N, Hellwinkel O, Schulz C, Chun FK, Huland H, Aepfelbacher M, Schlomm T: **Prevalence of human gammaretrovirus XMRV in sporadic prostate cancer.** *J Clin Virol* 2008, **43**:277-283.
7. Hohn O, Krause H, Barbarotto P, Niederstadt L, Beimforde N, Denner J, Miller K, Kurth R, Bannert N: **Lack of evidence for xenotropic murine leukemia virus-related virus (XMRV) in German prostate cancer patients.** *Retrovirology* 2009, **6**:92.
8. Lombardi VC, Ruscetti FW, Das Gupta J, Pfost MA, Hagen KS, Peterson DL, Ruscetti SK, Bagni RK, Petrow-Sadowski C, Gold B, Dean M, Silverman RH, Mikovits JA: **Detection of an infectious Retrovirus, XMRV, in Blood Cells of Patients with Chronic Fatigue Syndrome.** *Science* 2009, **326**:585-589.
9. Fukuda K, Straus SE, Hickie I, Sharpe MC, Dobbins JG, Komaroff A: **The chronic fatigue syndrome: a comprehensive approach to its definition and study. International Chronic Fatigue Syndrome Study Group.** *Ann Intern Med* 1994, **121**:953-959.
10. Mothes W, Boerger AL, Narayan S, Cunningham JM, Young JA: **Retroviral entry mediated by receptor priming and low pH triggering of an envelope glycoprotein.** *Cell* 2000, **103**:679-689.
11. Bock M, Bishop KN, Towers G, Stoye JP: **Use of a transient assay for studying the genetic determinants of Fv1 restriction.** *J Virol* 2000, **74**:7422-7430.
12. Temperton NJ, Hoschler K, Major D, Nicolson C, Manvell R, Hien VM, Ha do Q, de Jong M, Zambon M, Takeuchi Y, Weiss RA: **A sensitive retroviral pseudotype assay for influenza H5N1-neutralizing antibodies.** *Influenza Other Respi Viruses* 2007, **1**:105-112.
13. Chesebro B, Britt W, Evans L, Wehrly K, Nishio J, Cloyd M: **Characterization of monoclonal antibodies reactive with murine leukemia viruses: use in analysis of strains of Friend MCF and Friend ecotropic murine leukemia virus.** *Virology* 1983, **127**:134-148.
14. Evans LH, Morrison RP, Malik FG, Portis J, Britt WJ: **A neutralizable epitope common to the envelope glycoproteins of ecotropic, polytropic, xenotropic, and amphotropic murine leukemia viruses.** *J Virol* 1990, **64**:6176-6183.
15. Hickie I, Davenport T, Vernon SD, Nisenbaum R, Reeves WC, Hadzi-Pavlovic D, Lloyd A: **Are chronic fatigue and chronic fatigue syndrome valid clinical entities across countries and health-care settings?.** *Aust N Z J Psychiatry* 2009, **43**:25-35.
16. Kato K, Sullivan PF, Evengard B, Pedersen NL: **A population-based twin study of functional somatic syndromes.** *Psychol Med* 2009, **39**:497-505.
17. Kerr JR, Petty R, Burke B, Gough J, Fear D, Sinclair LI, Mattey DL, Richards SC, Montgomery J, Baldwin DA, Kellam P, Harrison TJ, Griffin GE, Main J, Enlander D, Nutt DJ, Holgate ST: **Gene expression subtypes in patients with chronic fatigue syndrome/myalgic encephalomyelitis.** *J Infect Dis* 2008, **197**:1171-1184.
18. Erlwein O, Kaye S, McClure MO, Weber J, Wills G, Collier D, Wessely S, Cleare A: **Failure to Detect the Novel Retrovirus XMRV in Chronic Fatigue Syndrome.** *PLoS ONE* 2010, **5**:e8519.
19. Rosenberg N, Jolicoeur P: **Retroviral pathogenesis.** *Retroviruses* Cold Spring Harbor Press, Cold Spring Harbor, NYCoffin JM, Hughes SH, Varmus HE 1997, Chapter 10:475-585.

doi:10.1186/1742-4690-7-10
Cite this article as: Groom *et al.*: Absence of xenotropic murine leukaemia virus-related virus in UK patients with chronic fatigue syndrome. *Retrovirology* 2010 7:10.

BMJ

RESEARCH

Prevalence of xenotropic murine leukaemia virus-related virus in patients with chronic fatigue syndrome in the Netherlands: retrospective analysis of samples from an established cohort

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Cite this as: *BMJ* 2010;340:c1018
doi:10.1136/bmj.c1018

ABSTRACT

Objective The presence of the retrovirus xenotropic murine leukaemia virus-related virus (XMRV) has been reported in peripheral blood mononuclear cells of patients with chronic fatigue syndrome. Considering the potentially great medical and social relevance of such a discovery, we investigated whether this finding could be confirmed in an independent European cohort of patients with chronic fatigue syndrome.

Design Analysis of a well defined cohort of patients and matched neighbourhood controls by polymerase chain reaction.

Setting Certified (ISO 15189) laboratory of clinical virology in a university hospital in the Netherlands.

Population Between December 1991 and April 1992, peripheral blood mononuclear cells were isolated from 76 patients and 69 matched neighbourhood controls. In this study we tested cells from 32 patients and 43 controls from whom original cryopreserved phials were still available.

Main outcome measures Detection of XMRV in peripheral blood mononuclear cells by real time polymerase chain reaction assay targeting the XMRV *integrase* gene and/or a nested polymerase chain reaction assay targeting the XMRV *gag* gene.

Results We detected no XMRV sequences in any of the patients or controls in either of the assays, in which relevant positive and negative isolation controls and polymerase chain reaction controls were included. Spiking experiments showed that we were able to detect at least 10 copies of XMRV sequences per 10^5 peripheral blood mononuclear cells by real time as well as by nested polymerase chain reaction, demonstrating high sensitivity of both assays.

Conclusions This study failed to show the presence of XMRV in peripheral blood mononuclear cells of patients with chronic fatigue syndrome from a Dutch cohort. These data cast doubt on the claim that XMRV is associated with chronic fatigue syndrome in the majority of patients.

INTRODUCTION

Chronic fatigue syndrome, also named myalgic encephalitis, is characterised by disabling physical and mental fatigue, lasting for at least six months, without an apparent physical cause.[1-3] The hallmark of the illness is debilitating fatigue, but symptoms like myalgia, disrupted sleep, difficulty with concentration, sore throat, and lymphadenopathy may also be present, albeit more variably. More than two thirds of patients are women. Although the cause is unknown and the illness may cover more than one entity, many have suggested that infectious agents have a role.[4] Indeed, the onset of chronic fatigue syndrome is often preceded by an acute flu-like illness or infectious mononucleosis with seemingly impaired recovery.[5] A role of chronic infection and changed immunity has been postulated. Most cases of the illness are sporadic, but some clustered cases have been described, particularly suggesting an infectious cause. However, despite extensive studies, no causative infectious agent has been conclusively identified, neither has an immune defect been established to explain the symptoms.[2,6]

In a recent publication in *Science*, Lombardi et al[7] reported the detection of xenotropic murine leukaemia virus-related virus (XMRV)—a human gamma retrovirus that was first identified in tumour tissue of patients with prostate cancer[8]—in peripheral blood mononuclear cells of patients with chronic fatigue syndrome. In that study, XMRV was detected by polymerase chain reaction in 67% of patients (68 of 101 samples) and in 4% of healthy individuals (eight of 213 samples). Furthermore, antibodies to XMRV were identified in the blood of patients but not in controls. Lombardi et al showed that XMRV was infectious and transmittable from clinical material of patients to T cell cultures and a

permissive cell line. The genetic sequence of XMRV in patients was nearly identical to that in patients with prostate cancer, indicating that the identified retrovirus is a genuine human virus rather than a mouse leukaemia virus contamination.

This report was considered a major scientific breakthrough and attracted a lot of attention. However, the paper fell short in the description of the patients: what was the nature of the cohort, what was the age and sex distribution, how well were the controls matched? Investigation of an independent cohort is therefore necessary before a causal association between XMRV infection and the development of chronic fatigue syndrome can be ascertained. We investigated the presence of XMRV in a well established Dutch cohort of patients with chronic fatigue syndrome using previously described real time and nested polymerase chain reaction assays on two different target genes.[7-9]

METHODS

Patient cohort

All patients and controls examined in this study were part of a Dutch cohort of 298 patients, which has been described in detail.[10,11] All patients of this cohort fulfilled the Oxford criteria and reported severe, unexplained, debilitating fatigue of at least one year in duration.[12] The median duration of their symptoms was seven years (range 2-45 years). The enrolled patients came to our outpatient clinic twice in a three month period. On the second visit, each patient was accompanied by a neighbourhood control (who was selected by the patient) of the same sex and within two years of the same age. Patients and controls visited our clinic between December 1991 and April 1992. All patients underwent a physical examination and an extensive laboratory work-up and completed a set of questionnaires.[10] Blood samples were obtained from 76 patients (randomly chosen using a table of random numbers[10] from the 298 patients described above) and 69 matched neighbourhood controls. Blood samples were sent to the central laboratory of the blood transfusion service in Amsterdam, where peripheral blood mononuclear cells were isolated for a study of lymphocyte subsets and apoptosis.[11] After isolation, a fraction of the peripheral blood mononuclear cells was directly cryopreserved according to a standard protocol in a computerised device. Cells were aliquoted in phials and stored with 10% dimethyl sulfoxide at −196°C (liquid nitrogen) in a density of about 10^7 cells per ml. The quality of the storage conditions at the central laboratory of the blood transfusion service has been amply demonstrated by Jansen et al, who showed that peripheral blood mononuclear cells stored for 12 years remained fully viable and immunologically competent.[13]

In this study, we examined peripheral blood mononuclear cells of all patients (n=32) and controls (n=43) from whom original cryopreserved vials were still available. This group included 25 patients and their matched controls, as well as seven patients and 18 controls that were not matched to each other. The male to female ratio of the patient group that was tested in this study was 1:2. Average age of the male patients was 40.7 years (range 25-61) and of female patients was 40.5 years (25-67).

Nucleic acid isolation and copy DNA synthesis

Nucleic acid was isolated from 100 µl of peripheral blood mononuclear cells (about 0.5 to 2×10^6 cells) using the MagNA-Pure LC and the MagNA-Pure LC Total Nucleic Acid Isolation Kit (Roche Diagnostics, Almere, Netherlands) according to the instructions of the manufacturer and eluted in 50 µl of elution buffer. A fixed amount of phocine distemper virus, a paramyxovirus that was used as internal control, was added to the samples before nucleic acid isolation so that we could monitor RNA quality and possible inhibition of amplification of the samples.[14] RNA in the total nucleic acid isolates was reverse transcribed to copy DNA using the TaqMan Reverse Transcription Reagents kit (Applied Biosystems, Nieuwerkerk aan den IJssel, Netherlands) in a 50 µl reaction mix containing 20 µl of nucleic acid isolate (concentration 25-150 ng per µl) and random hexamers as primers, according to the manufacturer's instructions.

Real time polymerase chain reaction assay

A duplex real time polymerase chain reaction assay was developed, adapted from the XMRV *integrase* real time polymerase chain reaction assay described by Schlaberg et al,[9] to detect XMRV and phocine distemper virus simultaneously. The reaction mixture contained 12.5 µl of 2X LightCycler 480 Probes Master (Roche Diagnostics), 1 µM of each primer and 400 nM of each probe, and 5 µl of copy DNA in a reaction volume of 25 µl. The XMRV and phocine distemper virus primers were as described.[9,14] The XMRV probe was used as a 5′-(6-carboxyfluorescein)-labelled, locked nucleic acid hydrolysis probe and the phocine distemper virus probe was used as a 5′-yakima yellow-labelled, locked nucleic acid hydrolysis probe. All primers and probes used in this study are shown in the table. Cycling conditions were 95°C for five minutes, followed by 50 cycles of 95°C for 15 seconds and 60°C for 45 seconds using the LightCycler 480 instrument (Roche Diagnostics). The result of the sample was considered a valid result only if the crossing point value for the spiked phocine distemper virus was within two cycles of the average of uninhibited samples.

Positive and negative controls for isolation, reverse transcription, and polymerase chain reaction were included in each run. β-globin real time polymerase chain reaction was performed using primers and hybridisation probes as described.[15] Mean crossing point value of β-globin real time polymerase chain reaction assay was 23.84, standard deviation 0.95. As a positive control for the polymerase chain reaction assay, we used nucleic acid isolated from 22Rv1, a prostate carcinoma cell line (American Type Culture Collection number CRL-2505) that was recently shown to contain multiple integrated copies of XMRV and to produce

high levels of infectious virus.[16] Total nucleic acid isolation and sample preparation from this cell line was as described above for peripheral blood mononuclear cells.

To determine the sensitivity of the XMRV real time polymerase chain reaction assay, we generated a 192 base pairs XMRV *integrase* polymerase chain reaction product using primers XMRV-F2 (which is located upstream of XMRV-F1) and XMRV-R3 (which is located downstream of XMRV-R2), and 22Rv1 copy DNA as a template. The polymerase chain reaction product was purified using the Wizard PCR preps DNA purification system (Promega Benelux, Leiden, Netherlands). The concentration was determined using a NanoDrop 1000 (Thermo Scientific/Isogen, De Meern, Netherlands) and the number of copies per µl was calculated. A dilution series was made in which 10^1 to 10^7 copies of the calibrator were added to 10^6 peripheral blood mononuclear cells before nucleic acid isolation. This corresponds to 1 to 10^6 copies per reaction, since a tenth of the isolated nucleic acid was used as input for the polymerase chain reaction, which was performed as described above.

Nested polymerase chain reaction assay

The XMRV *gag* nested polymerase chain reaction assay was adapted from Urisman et al.[8] The reaction mixtures contained 25 µl of 2X PCR Master (Roche Diagnostics), and 200 nM of each primer in a reaction volume of 50 µl. In the first reaction, 5 µl of copy DNA was used. Subsequently, 5 µl of the first reaction was used as input for the nested reaction. Primers were as described, except for the reverse primer of the nested reaction (GAG-I-R), which was replaced by GAG-I-R2 to yield a 92 base pairs reaction product (we used primer GAG-I-R2 because it produced less background in the nested reverse transcription polymerase chain reaction). The target sequence of GAG-I-R2 is 100% conserved among all XMRV isolates published to date (data not shown). Cycling conditions were as previously described.[8] Polymerase chain reaction products (20 µl) were analysed on a 2.5% agarose gel.

To determine the sensitivity of our XMRV nested polymerase chain reaction assay, a 708 base pairs XMRV *gag* polymerase chain reaction product was generated using primers GAG-UNIQ-F described by Dong et al[17] (which is located upstream of GAG-O-F) and GAG-O-R, and 22Rv1 copy DNA as a template. Purification and determination of the amount of the polymerase chain reaction product were performed as described above for the real time polymerase chain reaction calibrator. A dilution series was made and 10^1 to 10^7 copies of the calibrator were added to 10^6 peripheral blood mononuclear cells prior to nucleic acid isolation. This corresponds to 1 to 10^6 per reaction since a tenth of the isolated nucleic acid was used as input for the nested polymerase chain reaction, which was performed as described above. In the same way, we tested the sensitivity of the nested polymerase chain reaction assay described by Urisman et al[8] using primer GAG-I-R instead of GAG-I-R2.

Sequences of primers and probes used in this study

	Sequence	Reference
XMRV integrase gene		
XMRV-F1	5'-CGAGAGGCAGCCATGAAGG-3'	9
XMRV-F2	5'-AACCTGATGGCAGATCAAGC-3'	This study
XMRV-R1	5'-GAGATCTGTTTCGGTGTAATGGAAA-3'	9
XMRV-R2	5'-CCCAGTTCCCGTAGTCTTTTGAG-3'	9
XMRV-R3	5'-TTTGCCTTGTAGGACCCAAT-3'	This study
XMRV-Probe	5'-AGTTCTAGAAACCTCTACACTC- 3'	9
XMRV gag gene		
GAG-O-F	5'-CGCGTCTGATTTGTTTTGTT-3'	8
GAG-O-R	5'-CCGCCTCTTCTTCATTGTTC-3'	8
GAG-I-F	5'-TCTCGAGATCATGGGACAGA-3'	8
GAG-I-R	5'-AGAGGGTAAGGGCAGGGTAA-3'	8
GAG-I-R2	5'-CAGACTGGTTGGATGCAATG-3'	This study
GAG-UNIQ-F	5'-GACTTTTTGGAGTGGCTTTGT-3'	17
PDV haemagglutinin gene		
PDV-F	5'-GGTGGGTGCCTTTTACAAGAAC-3'	14
PDV-R	5'-ATCTTCTTTCCTCAACCTCGTCC-3'	14
PDV-probe	5'-ATGCAAGGGCCAATT-3'	14
Human β-globin gene		
bGLO-for	5'-GAGCCATCTATTGCTTACATTTGC-3'	15
bGLO-rev	5'-TTGGTCTCCTTAAACCTGTCTTGT-3'	15
bGLO-FL	5'-CCAGGGCCTCACCACCAACTTC-3'	15
bGLO-LC640	5'-CCACGTTCACCTTGCCCCACAG-3'	15

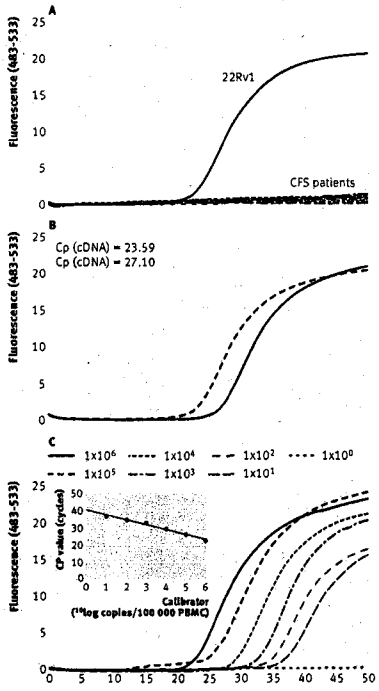

Fig 1| Results of XMRV *integrase* real time polymerase chain reaction assay. (A) All 32 patients with chronic fatigue syndrome (CFS) compared with positive 22Rv1 control, which yielded a crossing point value of about 23. Results for neighbourhood controls not shown. (B) 22Rv1 total nucleic acid (DNA, solid), reverse transcribed total nucleic acid (cDNA, dashed). The additional reverse transcription step increased the sensitivity of the polymerase chain reaction, decreasing the crossing point (Cp) value by 3.5. One of three independent experiments is shown. (C) Sensitivity of the assay. The inlay shows linear relation between number of spiked molecules and crossing point value from 10^1 to 10^6 copies per reaction

RESULTS

Total nucleic acid was isolated from peripheral blood mononuclear cells of 32 patients and 43 healthy controls. Nucleic acid was subjected to copy DNA synthesis to increase the sensitivity of our polymerase chain reaction assays. This was done because we observed that the real time polymerase chain reaction assay on nucleic acid isolated from a XMRV positive prostate cancer cell line, 22Rv1, and subjected to copy DNA synthesis—allowing detection of both proviral DNA and viral RNA—was about 10 times more sensitive than without copy DNA synthesis (fig 1B). Nevertheless, all samples from patients with chronic fatigue syndrome and from controls tested negative for both the XMRV *integrase* gene (fig 1) and the XMRV *gag* gene (fig 2).

Our negative XMRV polymerase chain reaction results were unlikely to be due to low amounts of nucleic acid tested or low sensitivity of the assays used. We used 50-300 ng of total nucleic acid from peripheral blood mononuclear cells per polymerase chain reaction, which is similar to the amount used by Lombardi et al.[7] Moreover, by adding 10-fold serial dilutions of a defined amount of template DNA to peripheral blood mononuclear cells before nucleic acid isolation, we demonstrated that both the real time polymerase chain reaction assay (fig 1C) and the nested polymerase chain reaction assay (fig 2B) could detect at least 10 copies of XMRV per 10^5 peripheral blood mononuclear cells, indicating a high sensitivity. A similar sensitivity of the nested polymerase chain reaction assay was observed when we used the same primers as described by Urisman et al (inner reverse primer GAG-I-R instead of the inner reverse primer GAG-I-R2 used in our assay) (data not shown).[8]

Our negative XMRV polymerase chain reaction results are also unlikely to be due to problems with nucleic acid isolation, loss of RNA or DNA integrity, synthesis of copy DNA, or the polymerase chain reaction procedure, since both the phocine distemper virus RNA (an internal control of which a fixed amount was added to each of the samples before nucleic acid isolation) and the β-globin gene were efficiently amplified in all samples tested (data not shown).

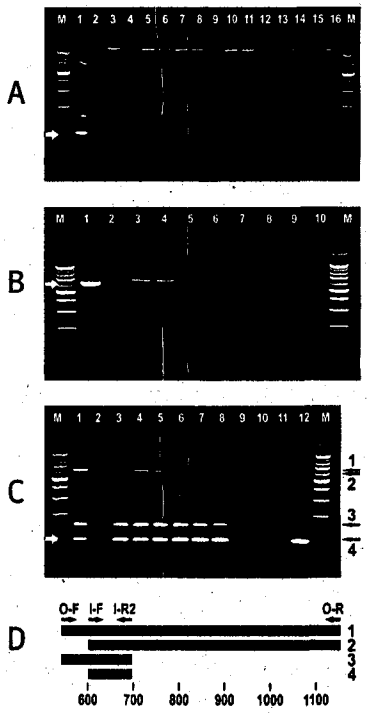

Fig 2| Results of XMRV *gag* nested polymerase chain reaction assay. (A) Results for 11 patients with chronic fatigue syndrome and negative controls. Results for neighbourhood controls not shown. (1) The positive 22Rv1 control yielded a product of the expected size of 92 base pairs (arrow); (2) negative polymerase chain reaction control; (3) phocine distemper virus (internal control); (4) negative reverse transcription control; (5)-(11) and (13)-(16) patient samples; (12) negative isolation control; (M) 100 base pairs size marker. (B) Sensitivity of the XMRV *gag* first polymerase chain reaction. (C) Sensitivity of the nested reaction. White arrows indicate the 613 base pairs (B) and 92 base pairs (C) reaction products. (1) 22Rv1; (2) negative polymerase chain reaction control; (3-9), dilution series of 10^6 to 10^0 copies of calibrator per reaction; (10) negative isolation control; (11) negative nested polymerase chain reaction control; (12) positive nested polymerase chain reaction control (22Rv1); (M) 100 base pairs size marker. Black arrows 1-4 in (C) indicate polymerase chain reaction products that are formed in the nested reaction (see D). (D) Positions of the *gag* primers and the *gag* polymerase chain reaction products formed in the nested reaction. In addition to the primers of the nested reaction (GAG-I-F, I-F and GAG-I-R2, I-R2), primers from the first reaction (GAG-O-F, O-F and GAG-O-R, O-R) are also present in the nested reaction, yielding reaction products 1-4 that correspond to the black arrows in (C). Numbers represent the positions on the XMRV genome (VP42, accession DQ241302)

WHAT IS ALREADY KNOWN ON THIS TOPIC

Chronic fatigue syndrome is a debilitating disease of unknown cause that affects millions of people worldwide

A study from the United States reported the detection of the retrovirus xenotropic murine leukaemia virus-related virus (XMRV) in peripheral blood mononuclear cells in a cohort of patients with chronic fatigue syndrome, suggesting a possible causal relation and a satisfactory explanation for their problems

WHAT THIS PAPER ADDS

We found no evidence for the occurrence of XMRV in peripheral blood mononuclear cells of patients with chronic fatigue syndrome from a well defined Dutch cohort

These data cast doubt on the claim that XMRV is associated with chronic fatigue syndrome in the majority of patients

DISCUSSION

Principal findings

We assessed the presence of XMRV in peripheral blood mononuclear cells isolated from patients with chronic fatigue syndrome from a well characterised Dutch cohort. We found no evidence for the presence of XMRV in any of these sporadic cases of chronic fatigue syndrome or in controls.

Strengths and limitations of the study

A limitation of our study is that the numbers of patients and controls in our study were relatively small. Based on these low numbers, the upper limit of the 95% confidence interval is a prevalence of 9% for the patient group and 7% for the control group, as calculated according to Eypasch et al (by the formula p=3/n).[18] Although we cannot formally rule out a role of XMRV, our data cast doubt on the claim that this virus is associated with chronic fatigue syndrome in the majority of patients.

Comparison with findings of previous studies

The results of the present study are in contrast with the findings of Lombardi et al, who detected XMRV in 67% of the patients with chronic fatigue syndrome analysed.[7] Technical aspects are unlikely to explain the difference in XMRV positivity rate between our data and their data. The possibility that the relative long duration of chronic fatigue syndrome in our cohort may have led to our negative results seems unlikely, because retroviruses integrate into the genome of the host. Given the high sensitivity of our real time and nested polymerase chain reaction assays, a positive signal should have been obtained in the presence of the virus. The fact that our samples were cryopreserved for many years is also unlikely to account for the negative results. Peripheral blood mononuclear cells cryopreserved for 12 years under these conditions have remained viable and immunocompetent.[13] Moreover, we found no difference in efficiency of β-globin gene amplification from stored samples compared with samples that were used directly after isolation (data not shown), indicating good quantity and quality of the nucleic acid isolated from cryopreserved samples.

As technical aspects do not seem to provide an explanation, the difference might be explained by the two cohorts studied. Our patients met the Oxford criteria for chronic fatigue syndrome, whereas the patients studied by Lombardi et al were reported to fulfil the Centers for Disease Control criteria,[7] but this is unlikely to explain the absence of XMRV in our patients' samples. Unfortunately, the paper of Lombardi and colleagues lacked a clear description of their patient cohort. Recently, at the Tri-Society Annual Conference 2009 in Lisbon, a presentation reported that the peripheral blood mononuclear cells were derived from patients from the outbreak of chronic fatigue syndrome at Incline village at the northern border of Lake Tahoe, United States (1984-5).[10] This outbreak has long been thought to have been caused by a viral infection and has been associated with a number of viruses, most notably Epstein-Barr virus[20] and human herpes virus 6,[21] but firm evidence for a role of viruses in this particular outbreak has never been provided. It is possible that the study of Lombardi et al has unravelled the viral cause of the chronic fatigue syndrome outbreak, but it seems unlikely that their study demonstrates a viral association for sporadic chronic fatigue syndrome cases, such as those we tested, or represents the majority of patients. Studies of XMRV in sporadic chronic fatigue syndrome cases from the United States would be of great interest.

XMRV was initially identified in tumour tissue of about 10% of patients with prostate cancer in the United States.[8] This association was recently confirmed in another independent study from the United States, in which XMRV was detected in 23% of patients.[9] Remarkably, in three independent European cohorts of patients with prostate cancer, no XMRV was detected.[22-24] Whether this discrepancy is due to differences in the geographic distribution of the virus remains to be established.

Recently, a team from the United Kingdom reported the failure to detect XMRV in all 186 tested peripheral blood mononuclear cell samples from a well characterised cohort of British patients with chronic fatigue syndrome.[25] This team, however, did not use the same primer sets as used by Lombardi et al, leaving open a possible explanation for the difference in results. In our study, we used the same primer sets as used by Lombardi et al. Although our patient group was relatively small and more research is required, our findings—together with those of Erlwein et al[25]—cast doubt on the claim that XMRV is associated with chronic fatigue syndrome in the majority of patients.

Lombardi et al also detected XMRV in about 4% of healthy controls.[7] We failed to detect XMRV in peripheral blood mononuclear cells of healthy controls in our study, but the number of controls tested (n=43) is too low to exclude the occurrence of XMRV in blood in a part of the population. Clearly, more research is needed to establish the distribution of XMRV in healthy controls and, of course, in blood supply products in Europe and in the United States.

Implications

In conclusion, we found no evidence for a role of XMRV in the cause of chronic fatigue syndrome in Dutch patients. Over the past decades we have seen a series of papers prematurely claiming the discovery of the microbial cause of chronic fatigue syndrome. Regrettably, thus far none of these claims has been substantiated.

Contributors: FJMvK, MGN, JMDG, and JWMvdM designed the study and wrote the paper. ASdJ, KHL, GWV, and WJGM performed experiments and analysed the data. CMAS, GB, JMDG, and JWMvdM established the chronic fatigue syndrome patient cohort. All authors had full access to all of the data (including statistical reports and tables) in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. FJMvK and JWMvdM are guarantors of the paper and accept full responsibility for the work and/or the conduct of the study, had access to the data, and controlled the decision to publish.
Funding: None.
Competing interests: All authors have completed the Unified Competing Interest form at www.icmje.org/coi_disclosure.pdf (available on request from the corresponding author) and declare that none of them (1) has support from companies for the submitted work; (2) has relationships with companies that might have an interest in the submitted work in the previous 3 years; (3) has spouses, partners, or children that have financial relationships that may be relevant to the submitted work, and (4) has non-financial interests that may be relevant to the submitted work.
Ethical approval: Ethical aspects of this study were approved by the Commissie Mensgebonden Onderzoek from Radboud University Medical Centre (CMO-1991).
Data sharing: No additional data available.

1 Fukuda K, Straus SE, Hickie I, Sharpe MC, Dobbins JG, Komaroff A. The chronic fatigue syndrome: a comprehensive approach to its definition and study. International Chronic Fatigue Syndrome Study Group. *Ann Intern Med* 1994;121:953-9.
2 Prins JB, van der Meer JW, Bleijenberg G. Chronic fatigue syndrome. *Lancet* 2006;367:346-55.
3 Cho HJ, Skowera A, Cleare A, Wessely S. Chronic fatigue syndrome: an update focusing on phenomenology and pathophysiology. *Curr Opin Psychiatry* 2006;19:67-73.
4 Swartz MN. The chronic fatigue syndrome - one entity or many? *N Engl J Med* 1988;319:1726-8.
5 White PD, Thomas JM, Kangro HO, Bruce-Jones WDA, Amess J, Crawford DH, et al. Predictions and associations of fatigue syndromes and mood disorders that occur after infectious mononucleosis. *Lancet* 2001;358:1946-54.
6 Lyall M, Peakman M, Wessely S. A systematic review and critical evaluation of the immunology of chronic fatigue syndrome. *J Psychosom Res* 2003;55:79-90.
7 Lombardi VC, Ruscetti FW, Das Gupta J, Pfost MA, Hagen KS, Peterson DL, et al. Detection of an infectious retrovirus, XMRV, in blood cells of patients with chronic fatigue syndrome. *Science* 2009;326:585-9.
8 Urisman A, Molinaro RJ, Fischer N, Plummer SJ, Casey G, Klein EA, et al. Identification of a novel Gammaretrovirus in prostate tumors of patients homozygous for R462Q RNASEL variant. *PLos Pathog* 2006;2:211.
9 Schlaberg R, Choe DJ, Brown KR, Thaker HM, Singh IR. XMRV is present in malignant prostatic epithelium and is associated with prostate cancer, especially with high-grade tumors. *Proc Natl Acad Sci USA* 2009;106:16351-56
10 Vercoulen JH, Swanink CM, Fennis JF, Galama JM, van der Meer JW, Bleijenberg G. Dimensional assessment of chronic fatigue syndrome. *J Psychosom Res* 1994;38:383-92.
11 Swanink CM, Vercoulen JH, Galama JM, Roos MT, Meyaard L, van der Ven-Jongekrijg J, et al. Lymphocyte subsets, apoptosis, and cytokines in patients with chronic fatigue syndrome. *J Infect Dis* 1996;173:460-3.
12 Sharpe MC, Archard LC, Banatvala JE, Borysiewicz, LK, Clare AW, David A, Edwards RH, et al. A report—chronic fatigue syndrome: guidelines for research. *J R Soc Med* 1991;84:118-21.
13 Jansen CA, de Cuyper IM, Hoolbrink B, van der Bij AK, van Baarle D, Miedema F. Prognostic value of the HIV-I gag-specific CD4+ T-cell responses for progression to AIDS analyzed in a prospective cohort study. *Blood* 2006;107:1427-33.
14 Clancy A, Crowley B, Niesters H, Herra C. The development of a qualitative real time RT-PCR for the detection of hepatitis C virus. *Eur J Clin Microbiol Infect Dis* 2008;27:1177-82.
15 Hesselink AT, van den Brule AJ, Groothuismink ZM, Molano M, Berkhof J, Meijer CJ, et al. Comparison of three different PCR methods for quantifying human papillomavirus type 16 DNA in cervical scrape specimens. *J Clin Microbiol* 2005;43:4868-71.
16 Knouf EC, Metzger MJ, Mitchell PS, Arroyo JD, Chevillet JR, Tewari M, et al. Multiple integrated copies and high-level production of the human retrovirus XMRV (xenotropic murine leukemia virus-related virus) from 22Rv1 prostate carcinoma cells. *J Virol* 2009;83:7353-6.
17 Dong B, Kim S, Hong S, Das Gupta J, Malathi K, Klein E, et al. An infectious retrovirus susceptible to an IFN antiviral pathway from human prostate tumors. *Proc Natl Acad Sci USA* 2007;104:1655-60.
18 Eypasch E, Lefering R, Kum CK, Troidl H. Probability of adverse events that have not yet occurred: a statistical reminder. *BMJ* 1995;311:619-20.
19 Mikovits J. Detection and immune correlates of an infectious retrovirus, XMRV, in blood cells of patients with chronic fatigue syndrome. Lisbon: Tri-Society Annual Conference, 18-21 October 2009. *Cytokine* 2009;48:10.
20 Holmes GP, Kaplan JE, Stewart JA, Hunt B, Pinsky PF, Schonberger LB. A cluster of patients with a chronic mononucleosis-like syndrome. Is Epstein-Barr virus the cause? *JAMA* 1987;257:2297-302.
21 Daugherty SA, Henry BE, Peterson DL, Swarts RL, Bastien S, Thomas RS. Chronic fatigue syndrome in northern Nevada. *Rev Infect Dis* 1991;13(Suppl 1):s39-44.
22 Fisher N, Hellwinkel O, Schulz C, Chun FK, Huland H, Aepfelbacher M, Schlomm T. Prevalence of human gammaretrovirus XMRV in sporadic prostate cancer. *J Clin Virol* 2008;43:277-83.
23 Hohn O, Krause H, Barbarotto P, Niederstadt L, Beimforde N, Denner J, Miller K, et al. Lack of evidence for xenotropic murine leucemia virus-related virus (XMRV) in German prostate cancer patients. *Retrovirology* 2009;6:92.
24 D'Arcy F, Foley R, Perry A, Marignol L, Lawler M, Gaffney E, et al. No evidence of XMRV in Irish prostate cancer patients with the R462Q mutation. *Eur Urol* 2008;Suppl 7:271.
25 Erlwein O, Kaye S, McClure M, Weber J, Wills G, Collier D, et al. Failure to detect the novel retrovirus XMRV in chronic fatigue syndrome. *PLoS ONE* 2010;5:e8519.

Accepted: 16 February 2010