The Euroline package insert quotes a study of the line blot giving an estimated false positive rate of 4% for HMGR autoantibodies in a population of healthy blood donors ($n=120$). In a comparison study of three other technologies all using HMGR antigen adsorbed to various solid phases the false positive rate in healthy controls ($n=517$) was 0.6% whereas in a disease control group ($n=117$) it was 2.6%.⁹ The disease control group used in our study estimated the false positive rate for the line blot at 11.5%.

Immunoprecipitation in agarose maintains analytes in fluid phase. The method also better replicates the method originally used to make the association between HMGR autoantibodies and immune necrotising myositis.⁵ Whilst it is relatively labour intensive, agarose gel immunoprecipitation is not technically demanding. As the rate of positives is relatively low, additional testing can be relatively easily accommodated into laboratory workflows.

Clinicians working in peripheral centres have difficulty obtaining muscle biopsy. Pragmatically they place reliance on detection of autoantibodies in making a diagnosis. In doing so, they risk a common error of diagnostic reasoning: the inverse fallacy, i.e., the conditional probability (MSA positive given myositis) erroneously 'inverts' to (myositis given MSA positive).¹⁰ Therefore, it behoves adopting laboratories to apply as much rigor as is feasible to verify the manufacturer's quoted clinical decision limits^{4,11} as suitable for a laboratory's catchment. For low prevalence diseases such as autoimmune myositis this may prove challenging.

There are several limitations to this study. Firstly, only 12 of the 25 cases had undergone muscle biopsy. Eleven of the 12 biopsies reported a necrotising pattern and one reported features consistent with an inclusion body pattern (gel 2, patient 18). However, the latter patient was on statin therapy at disease onset, had a CK in the 12000s and responded to immunosuppressive therapy. Despite the biopsy report, the treating clinician considered the patient's disease was more in keeping with immune-necrotising myositis associated with statin use.

Secondly, the control group was made up of a heterogeneous sample of patient serum which may not reflect the type of patients within the diagnostic differential for myositis. Ideally for a diagnostic test, this control group should reflect the differential diagnosis of disease in patients referred from the local catchment population. An appropriate control group is critical in deciding the clinical decision limit that maximises diagnostic accuracy.

Finally, we acknowledge that interpretation of CIEP has an element of subjectivity and requires expertise in reading the gels.

Based on our study results we have introduced the new Euroline blot as our preferred screening method. Our current experience suggests that the clinical decision limit/cut-off for positivity on the HMGR line-blot lies between 10 and 30 AU but we are yet to decide if the local clinical decision limit for the HMGR antigen should differ from that recommended by the manufacturer. A two-tier testing system for HMGR autoantibodies testing has been adopted: screen by HMGR line blot with results >10 AU then tested for immunoprecipitating antibodies using CIEP. We and others¹² recommend a similar approach in all diagnostic laboratories.

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Ke Li Chow, Paula Elizabeth Keating, Myfanwy Bailey Spellerberg, Ignatius Chua, John Liston O'Donnell

Department of Immunology, Canterbury Health Laboratories, Christchurch, New Zealand

Contact Dr John Liston O'Donnell.

E-mail: john.odonnell@cdhb.health.nz

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Evidence of chromosomal damage in scleroderma

Sir,

Systemic sclerosis (scleroderma, SSc) is an uncommon heterogeneous connective tissue disease considered to have an autoimmune origin.¹ Cancer occurs more commonly in scleroderma² and we have previously documented acquired genetic damage at the glycophorin-A locus suggesting genomic instability.³

Seeking further evidence for genomic instability we examined peripheral blood lymphocytes (PBL) from scleroderma patients using the well validated and robust cytokinesis-

block micronucleus cytochrome (CBMN-cyt) assay. DNA damage is scored specifically in once-divided bi-nucleated cells and the damage index includes micronuclei (MNI), a biomarker of chromosome double strand breakage and/or whole chromosome loss, and nuclear buds (NBUDS), a biomarker of amplified DNA and/or unresolved DNA repair complexes.⁴ Micronutrient deficiencies, such as folate and vitamin B12, have also been linked with DNA damage events including MNI and NBUDS.⁵

We recruited 62 well-characterised SSc patients (age range 39–75 years, mean 57.43 years, SD 9.689) and 28 well-matched normal healthy controls (NHCs) (age range 29–85 years, mean 58.47 years, SD 11.503). Scleroderma patients had a mean age of onset ($\pm 95\%$ CI) = 41.7 ± 4.33 years and a mean disease duration ($\pm 95\%$ CI) = 17.5 ± 4.56 years. All SSc patients fulfilled the American Rheumatology Association’s 2013 SSc diagnostic criteria.¹ Subjects were excluded if they had previous or current cytotoxic drug exposure, radiotherapy, or current cancer. Ethics approval was obtained from each participating centre (approval number HREC/070405). Informed written consent was obtained from each participant in this study.

Serology for centromere antibody (CENP), anti-topoisomerase antibody (Topo1), RNA polymerase 3 antibody (RNAP3) and other antibodies (Table 1) were performed by indirect immunofluorescence, or line blot as previously described.⁶

Chromosomal damage was measured in once divided bi-nucleated cells (BNCs) in PBLs using the well-validated and robust CBMN-cyt assay as previously described.⁴ Nutritional biomarkers serum B12, serum folate and homocysteine were measured using standard techniques in a diagnostic biochemistry laboratory certified by the National Accreditation Testing Authority.

Results were analysed in SPSS v25 (IBM, USA) by analysis of variance (ANOVA) with pairwise comparisons and Fisher’s least significant difference post hoc testing.

Scleroderma patients identifying as CENP positive ($n=24$) had significantly higher levels of MNI than NHCs ($p=0.02$), or with patients with other autoantibodies (Topo1 $p=0.000$, RNAP3 $p=0.001$, other ($p=0.008$) (Fig. 1). CENP positive patients also had significantly higher NBUDS than NHCs ($p=0.000$) or with patients with other antibodies (Topo1 $p=0.000$, RNAP3 $p=0.029$, other $p=0.001$) (Table 2). A

Table 1 Autoantibody profile and disease subgroup status, cases and normal healthy controls

	Autoantibody				NHC (n)
	CENP (n)	Topo1 (n)	RNAP3 (n)	Other (n)	
NHC					23
Female					5
Male					
SSc					
Female					
Diffuse	0	4	6	5	
Limited	21	3	2	8	
Overlap	0	0	0	5	
Male					
Diffuse	0	2	1	1	
Limited	3	2	1	0	

CENP, centromere antibody; NHC, normal healthy controls; Other, other antibodies; RNAP3, RNA polymerase 3 antibody; SSc, scleroderma cases; Topo1, anti-topoisomerase antibody.

Fig. 1 Box and whisker plot. Number of micronuclei (MNI) in binucleated cells (BNCs) per 1000 BNCs, in normal healthy controls or case (scleroderma). Participants subdivided according to the presence of centromere antibody (CENP), topoisomerase antibody (Topo1) or RNA polymerase antibody (RNAP3) or other antibodies (Others).

positive regression trend was observed in the CENP subgroup between MNI and NBUDS ($R=0.39$, $p=0.06$). Other scleroderma serological subgroups showed no differences in DNA damage as compared with NHCs, and there was no difference between patients with limited and diffuse scleroderma, nor significant regression with disease duration ($R=0.24$, $p=0.18$). Furthermore, no significant differences were observed between NHCs and SSc for the three nutritional variables that are known to influence DNA damage biomarkers in the CBMN-cyt assay (serum B12, folate and homocysteine).⁷ No significant correlations were observed between the nutritional biomarkers and the MNI values.

The finding of significantly elevated MNI and NBUDS in the CENP positive subset of our scleroderma patients is of considerable interest and suggests the likelihood of numerical or structural chromosomal instability in this subset. MNI are a biomarker of chromosomal breaks and/or mal-segregation and have been shown to be elevated within peripheral blood lymphocytes and fibroblasts from patients with several clinical disorders, e.g., cancer, autoimmunity, Alzheimer’s disease, and malnutrition.⁸ Further, DNA trapped in MNI may trigger an auto-immune response via the innate immune receptor cyclic GMP-AMP synthetase (cGAS) which senses mislocalised endogenous nucleic acid; upon binding to double-stranded DNA, cGAS produces the second messenger cyclic GMP-AMP (cGAMP) that is recognised by the adaptor protein stimulator-of-interferon-genes (STING), resulting in type I interferon and proinflammatory cytokine production.⁹ With production of numerous pro-inflammatory cytokines, auto-immune activation may occur.¹⁰

Finding evidence of enhanced chromosomal damage in the CENP positive SSc subgroup was not anticipated because this variant has a more benign clinical phenotype compared with Topo1 and RNAP3 subgroups. However, an Italian study in 2002 reported similar MNI findings to those in our study.¹¹

The explanation for the increased MNI frequency in the CENP subgroup is intriguing and a central question is whether the CENP antibody is a direct consequence of prior chromosomal damage or is, in contrast, a causative factor in its own right? Furthermore, it is intriguing that as $>70\%$ of MNI in females contain inactivated X chromosome¹² it raises

Table 2 DNA damage events: analysis of variance multiple comparisons between autoantibodies and with normal healthy control subjects

(I) Autoantibody	(J) Autoantibody	Mean difference (I–J) ^a	Std error ^b	Sig	Confidence interval	
					Lower bound	Upper bound
CENP	Topo1	17.878	4.57616	0.000	8.7808	29.9751
	RNAP3	16.0996	4.73046	0.001	6.6958	25.5035
	Other	10.5906	3.91879	0.008	2.8004	18.3809
	Control	11.2856	3.49612	0.002	4.3356	18.2358
Topo1	RNAP3	–1.7783	5.49139	0.747	–12.6848	9.1382
	Other	–7.2873	4.80989	0.133	–16.8491	2.2745
	Control	–6.5923	4.47225	0.144	–15.4828	2.2982
RNAP3	Other	–5.509	4.95692	0.270	–15.363	4.345
	Control	–4.814	4.63001	0.301	–14.0182	4.3902

Dependent variable: number of micronuclei in binucleated cells (BNCs)/1000 BNCs.

CENP, centromere antibody; Other, other antibodies; RNAP3, RNA polymerase 3 antibody; Topo1, anti-topoisomerase antibody.

^a Least significant difference, based on observed means.

^b The error term is mean square (error) = 157.957.

the possibility of a causal association with the known striking female predominance of CENP positive scleroderma.

Experimental strengths in this present study include (1) the fact that all samples were blinded for analysis, (2) the scientist performing the reading of the CBMN-cyt assay had no prior knowledge of the clinical status or origin of the blood sample, and did all the readings for both patients and controls, (3) the control group was well matched for age, gender, ethnicity and B-vitamin status, (4) the patient cohort was well characterised both clinically and serologically. Limitations included the relatively small numbers of patients and controls studied allowing the possibility of a type II error.

In conclusion, elevated levels of MNi and NBUDS were observed in the female predominant CENP positive scleroderma subgroup. These findings did not appear to be related to disease duration nor deficiency of serum vitamin B12 and/or folate. Other serological scleroderma subgroups did not show elevated levels of these DNA damage biomarkers. The high levels of MNi and/or NBUDS in this selective CENP positive subgroup support the hypothesis that chromosomal instability may be an additional risk factor for autoimmunity in women.

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K. A. Patterson¹, J. G. Walker², P. J. Roberts-Thomson¹, C. F. Bull³, M. Fenech⁴

¹Department of Immunology, Flinders Medical Centre, Bedford Park, SA, Australia; ²Rheumatology Unit, Flinders Medical Centre, Bedford Park, SA, Australia; ³CSIRO

Health and Nutrition, Adelaide, SA, Australia; ⁴University of South Australia, Adelaide, SA, Australia

Contact Dr Karen Patterson.

E-mail: karen.patterson@flinders.edu.au

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Cytological diagnosis of mesothelioma *in situ* versus invasive mesothelioma

Sir,

We read with interest the article by Pulford *et al.*¹ on mesothelioma *in situ*. One of the cases described in that paper