Prevalence and Clinical Significance of anti-DFS70 in ANA Positive Patients Undergoing Routine ANA Testing in a New Zealand Public Hospital.

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Abstract

The presence of antinuclear antibodies (ANA) in serum is the hallmark diagnostic test for most Systemic Autoimmune Rheumatic Disease (SARD). Anti-dense fine speckled (DFS) 70 is an autoantibody that produces a characteristic dense fine speckled pattern in the ANA HEp-2 indirect immunofluorescence (IIF) assay. Its clinical significance is not yet clear however it has been reported that these antibodies are more prevalent in healthy individuals and non-SARD patients than in SARD patients. Thus it is has been proposed that the presence of anti-DFS70 antibodies could be used to eliminate a SARD diagnosis. To date, there is no published data as to whether this can be applied to a New Zealand population, nor what the prevalence of these antibodies are in a New Zealand population. The DFS IIF pattern can be difficult to identify and most New Zealand diagnostic laboratories do not specifically test for the autoantibody, therefore it is likely that its presence is currently being under-reported.

The purpose of this research was to determine if New Zealand diagnostic laboratories should be specifically testing for anti-DFS70 antibodies and including the result in the laboratory report. Thus the principal objectives of this research were to (1) determine if current routine ANA testing methods are detecting anti-DFS70 antibodies, (2) determine the local prevalence of anti-DFS70 in ANA positive patients in a New Zealand public hospital population, (3) determine if the presence of anti-DFS70 is clinically significant in terms of a SARD diagnosis. Should anti-DFS70 prove to be a significant factor in terms of eliminating a SARD diagnosis, then a new ANA diagnostic algorithm would be proposed.

Samples tested were a consecutive series of routine ANA positive patient samples at a general public hospital, consisting of 100 each of SARD and non-SARD patients. In order to ensure the likelihood of anti-DFS70 detection, two ANA detection methods were used (IIF and enzyme linked immunosorbent assay (ELISA)). All positive ANA samples were tested for anti-DFS70 by chemiluminescence immunoassay (CIA).

Results showed that both the ANA IIF and ELISA assays are detecting anti-DFS70 antibodies. The prevalence of anti-DFS antibodies in SARD patients was 1% and in non-SARD patients was 7% and the difference between the two was statistically significant. In non-SARD patients anti-DFS70 was usually found in isolation with no

other specific ANAs present. There was a significant difference in the prevalence of anti-DFS70 according to ethnicity but not by age or sex.

In conclusion, the presence of anti-DFS70 antibodies, particularly when present alone without any other specific ANAs present, makes a SARD diagnosis highly unlikely. Therefore New Zealand diagnostic laboratories should be specifically testing for anti-DFS70 antibodies and including the result in the laboratory report. An appropriate interpretative comment should be included in the report as it is imperative that clinicians are aware of the significance of the anti-DFS70 result.

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Attestation of Authorship

"I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person (except where explicitly defined in the acknowledgements), nor material which to a substantial extent has been submitted for the award of any other degree or diploma of a university or other institution of higher learning."

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Ethics Approval

This study was given full ethical approval by the New Zealand Health and Disability Ethics Committee (HDEC) on 15 July 2015. Reference number 15/CEN/103 (see Appendix A). Full ethical approval was also given by the WDHB Ethics Committee as well as the WDHB Maori Ethics Committee.

1 Background / Literature Review

1.1 Antinuclear Antibodies

The term ‘antinuclear antibodies’ (ANA) originally referred to a group of autoantibodies directed against nuclear antigens, however it has since been shown that some ANAs are directed against antigens in the cell cytoplasm or membrane. Nevertheless ANAs continue to be the term used for these autoantibodies (Smeenk, 2000). There are many types of specific ANAs (e.g. anti-dsDNA, anti-Ro and anti-Scl70) also known as antibodies to extractable nuclear antigens (ENAs) (Aggarwal, 2014). The term ENA was first described in 1959 by Holman and Robbins for a group of nuclear antigens that were extractable by saline solutions. However since then insoluble nuclear antigens (such as double stranded DNA (dsDNA)) and cytoplasmic target antigens have been discovered (Mahler, Meroni, Bossuyt & Fritzler, 2014). Whilst this terminology is also now outdated, it is still commonly used worldwide. There have been calls to replace the ANA and ENA terminology with more appropriate ones. An international expert panel have suggested ‘anticellular antibodies’ and ‘specific antibodies’ respectively but as yet there have been no immediate moves to implement these changes (Agmon-Levin et al., 2014).

Traditionally, the presence of ANA in serum is considered as the hallmark diagnostic test for systemic autoimmune rheumatic diseases (SARD) such as systemic lupus erythematosus (SLE), Sjögren’s syndrome and systemic sclerosis, as elevated levels of ANA are usually seen in SARD conditions. However, the specificity and simplicity of the ANA test are questionable as a positive ANA does not automatically translate into a diagnosis of an autoimmune or connective tissue disease. Elevated levels of ANA can also be found in some cancers and infections as well as in healthy individuals. This can therefore complicate the interpretation and clinical application of the ANA test results (Copple, Jaskowski, Giles & Hill, 2014).

Some ENAs are highly specific for a SARD condition. For example, anti-dsDNA antibodies are highly specific for SLE and are present in about two-thirds of SLE patients. Anti-Sm antibodies are also highly specific for SLE. They appear in 15-30% of SLE patients but are very rarely seen other autoimmune diseases (Aggarwal, 2014). In addition, in some cases ANAs may appear in the blood for more than a year before

clinical symptoms of a SARD condition begin to manifest, which can further complicate the ANA result interpretation. For example, anti-dsDNA can appear in the blood for more than a year before any clinical signs and symptoms of SLE begin to show (Smeenk, 2000).

1.1.1 ANA testing overview

ANAs were first demonstrated in 1957 by Holborow et al. using indirect immunofluorescence (IIF) on organ tissue (Smeenk, 2000). Modern IIF assays now use cultured human epithelial tumour cell line (HEp-2) as a substrate (Mahler & Fritzler, 2012). The IIF assay can detect ANA autoantibodies to over 100 different nuclear and cytoplasmic antigens. Each clinical laboratory usually determines its own cut-off for a positive ANA IIF result, therefore there is variability between laboratories (Meirendorf & Shmerling, 2012).

In the ANA HEp-2 IIF assay, different types of ANAs produce different fluorescent staining patterns on the HEp-2 cells. The type of fluorescent pattern may help towards identifying the specific ANA present and the type of pattern may also correlate with certain diseases. However the patterns are not specific to a certain condition. For example, a speckled pattern is common in Sjögren's syndrome but can also be seen in other conditions such as SLE and systemic sclerosis (Aggarwal, 2014). While the ANA IIF pattern is usually presented in the laboratory report, it is rarely used for disease diagnosis and is not included in SARD disease classification criteria, including for SLE. The presence of any specific ANA is usually more useful than the type of pattern seen (Mahler et al., 2014).

Most clinical laboratories would then go on to perform an ANA titer on all ANA positive samples. Changes in titer over time may be used by clinicians to track the progress of the condition and/or monitor the effectiveness of a treatment (Copple et al., 2014). Although in some cases, such as for SLE, there is no evidence that changes in ANA titer correlate with disease activity (Meirendorf & Shmerling, 2012) therefore it is important that clinicians are aware of this in order to avoid unnecessary repeat testing of these patients.

Once a titer is performed, the ANA IIF positive samples are usually then further tested with more specific assays for the presence of most of the common ANAs. The ENA panel will usually include antibodies to dsDNA, Ro (SSA), La (SSB), Jo-1, Scl-70, Sm

and RNP; however other antibodies may also be included. There are currently various methodologies for ENA antibody detection and they include enzyme linked immunosorbent assay (ELISA), immunoblotting and more recently chemiluminescence immunoassay (CIA) (Aggarwal, 2014).

1.1.2 Limitations of the ANA HEp-2 assay

While the ANA HEp-2 IIF assay is highly sensitive, it does have some limitations. Firstly, it is a laborious process as titers require serial dilutions of patient sera and all slides require IIF pattern interpretation. Pattern interpretation is highly subjective and sometimes hard to interpret, therefore slide reading requires highly experienced scientists. These factors also add to the expense of the assay (Meroni, Bizzaro, Cavazzana, Borghi & Tincani, 2014). Other variables can also affect the IIF assay such as differences in the HEp-2 cell lines, conjugate and even the type of microscope or bulb used. These also all lead to a lack of standardisation of the assay across laboratories (Copple et al., 2014). Another limitation of the assay is its lack of specificity as there have been reports that the ANA IIF method can have false-positive rates as high as 20%. This results in unnecessary follow up testing and management of these patients. The high sensitivity and low specificity of the HEp-2 IIF assay is highlighted in the case of SLE, where it is reported to have a sensitivity of greater than 95%, but with a specificity of just 57% (Meroni et al., 2014).

Because of the above limitations of the ANA HEp-2 IIF assay, other methods for ANA detection have been developed in the hope to eliminate or reduce some of these limitations. The newer ANA detection methods include ELISA, EIA and multiplex assays. While these newer assays are cheaper, faster and eliminate the need for subjective interpretations, they are qualitative only and so do not report any patterns or titers (Aggarwal, 2014). There is also a concern that they cannot show comparable sensitivity to the IIF assay. For this reason the American College of Rheumatology formed a Task Force in 2007 to provide some guidelines on ANA testing. One of their recommendations was that the IIF assay remains the Gold Standard for ANA testing (American College of Rheumatology, 2009).

1.2 Anti-DFS70

Only a minority of ANAs have been well researched and have been shown to be associated with certain conditions whereas further studies are required to help to identify the remaining ANAs and determine their clinical significance (Abeles & Abeles, 2013). Anti-dense fine speckled (DFS)70 is one such ANA that has attracted much research in recent years due to the relatively common occurrence of the DFS IIF pattern in healthy people and in patients without any clinical evidence of SARD (Mahler & Fritzler, 2012).

1.2.1 The DFS70 antigen

Anti-DFS70 is an auto-antibody to the DFS70 antigen, which is a nuclear antigen. The DFS70 antigen is so named for its immunofluorescent pattern on HEp-2 cells and its molecular mass in immunoblot assays of 70kDa. The autoantigen was later identified as the lens epithelium-derived growth factor (LEDGF) and then as the DNA binding transcription coactivator p75, therefore the autoantibody is sometimes referred to as anti-DFS70/LEDGF or anti-DFS70/LEDGFp75 (Mahler & Fritzler, 2012). DFS70 is a common nuclear autoantigen that is expressed in all cells and has important biological functions such regulation of gene expression and cellular stress response. Thus, when exposed to environmental stresses such as UVB irradiation, alcohol, and certain viruses and drugs, DFS70 is upregulated in a variety of cells and tissues in response. The DFS70 antigen has also been shown to be a transcription co-activator for HIV integration and has also been found to be overexpressed in certain cancer cells and tumours (Basu, Sanchez & Casiano, 2015).

It is not yet clear why the DFS70 antigen has such a common autoantibody response. One theory is that since it is abundantly expressed in the nucleus, it might be that inflammation enhances its immunogenicity by exposing cryptic epitopes and thus stimulating an autoantibody response (Bizzaro et al., 2015). There is also some evidence that human leukocyte antigen (HLA) Class II genes influence the production of anti-DFS70 antibodies and that they are natural antibodies, although more research is required to confirm these associations (Muro, Ogawa, Sugiura, & Tomita, 2006). It has been suggested that a better understanding of the DFS70 antigen biology is the key to determining the significance of its autoantibody (Basu et al., 2015).

1.2.2 Clinical significance of anti-DFS70

The clinical significance of anti-DFS70 antibodies is not yet clear, although there is some circumstantial evidence that they could play either protective, pathogenic or sensor roles (Basu et al., 2015). Anti-DFS70 antibodies were originally discovered in patients with interstitial cystitis but have since been found in a variety of conditions including cancer, infectious diseases and some inflammatory conditions (Bizzaro et al., 2015) and is even seen in approximately 10% of apparently healthy people (Watanabe et al., 2004). Anti-DFS70 antibodies are usually IgG and have been found in high titers in healthy individuals, often as high 1:5120. However it has been suggested that perhaps their presence is indicative of an undetected chronic inflammatory response in these so called 'healthy' individuals (Basu et al., 2015). In terms of SARD, previous research has shown that anti-DFS70 antibodies are rarely found in these patients. Dellavance et al. (2005) screened over 10,000 routine ANA positive samples and they found that 37% showed the DFS pattern, with most of these in non-SARD patients. Since the primary purpose of the ANA screen is to identify autoantibodies for SARD diagnosis, it is important to investigate whether anti-DFS70 antibodies are related to SARD (Muro, Sugiura, Morita, & Tomita, 2008) in order to prevent numerous follow-up testing and/or unnecessary treatment for these patients (Mahler et al., 2012).

1.2.3 Prevalence of anti-DFS70

The prevalence of the DFS pattern and anti-DFS70 in SARD patients is very low, with most studies reporting frequencies of less than 10%. For example, a study conducted by Muro et al. (2008) showed that 4% of SARD patients had anti-DFS70 and in a recent study, Bizzaro et al. (2015) reported that 7.5% of SARD patients had anti-DFS70. In addition, when anti-DFS70 antibodies do appear in SARD patients, they usually appear with other SARD specific autoantibodies, as indicated in Table 1. This was further highlighted in the study by Muru et al. (2008) where seven SLE patients that had anti-DFS70 also had at least one other clinically significant ANA present. More recently, Mahler et al. (2012) reported that of the eight SLE patients that tested positive for anti-DFS70, all except one patient had other autoantibodies present. The patient with an isolated anti-DFS70 had been diagnosed as SLE due to other classification criteria and so the diagnosis was not dependent on the ANA result.

Table 1.

Concomitance of specific antinuclear autoantibodies in anti-DFS70 antibody positive patients with systemic autoimmune rheumatic disease (SARD).

Study	Antinuclear antibodies	Percentage with isolated anti-DFS70
Muro et al. (2008)	anti-SSA, anti-SSB, anti-Sm, anti-Scl70, anti-U1-RNP, anti-centromere, anti-ssDNA	21%
Bizzaro et al. (2015)	anti-SSA/Ro	0%
Dellavance et al. (2005)[a]	anti-SSA/Ro	7%
Lee et al. (2016)	anti-SSA, anti-SSB, anti-Sm, anti-Sm/RNP, anti-Scl70, anti-U1-RNP, anti-centromere, anti-dsDNA, anti-ribosomal P	5%
Mahler et al. (2012)	anti-SSA, anti-SSB, anti-Sm, anti-dsDNA, anti-U1-RNP	14%

Note. [a]concomitant with the DFS pattern, presence of anti-DFS70 not confirmed.

The American College of Rheumatology has provided eleven criteria for the diagnosis of SLE which include symptoms as well as diagnostic test results (Table 2). A patient should have at least four of the eleven criteria either serially or simultaneously in order to be diagnosed as SLE (American College of Rheumatology, 1997). Therefore an SLE diagnosis is not always dependant on the ANA result. The aforementioned studies indicate that the diagnosis of SARD was not necessarily related to the presence of anti-DFS70, but instead due to the other ANAs present. Further evidence for this can be seen in the longitudinal Watanabe et al. (2004) study, where approximately 10% of healthy people had isolated anti-DFS70 antibodies and these people did not end up with a SARD condition with a follow up after four years. Based on these observations it has been proposed that the presence of anti-DFS70 antibodies, particularly when present alone without any other specific ANA antibodies present, could be used to exclude a SARD diagnosis or at least infer that a SARD diagnosis is highly unlikely (Mahler et al., 2012).

Table 2.

American College of Rheumatology criteria for the classification of Systemic Lupus Erythematosus (American College of Rheumatology, 1997).

	Criterion
1	Malar rash
2	Discoid rash
3	Photosensitivity
4	Oral ulcers
5	Nonerosive arthritis
6	Pleuritis or pericarditis
7	Renal disorder
8	Neurologic disorder
9	Haematologic disorder
10	Immunologic disorder
11	Positive antinuclear antibody

It appears that more research is required in order to confirm that the presence of anti-DFS70 may be used as a differential diagnosis for SARD. For example, a follow up of the Watanabe et al., (2004) study mentioned above, post 10 or 20 years later would be useful, as would other similar studies on healthy individuals. Mahler et al. (2012) also included healthy individuals in their study and they found that 9% of these healthy individuals had anti-DFS70 antibodies present, which is similar to the findings of the Watanabe study. However to date there has been no follow up testing on these patients so it is not known as to whether any of them did eventually develop a SARD condition. A recent paper by Gundin et al. (2016) showed that patients in their cohort with an isolated anti-DFS70 did not have SARD nor did they develop a SARD condition when followed up 10 years later. The results of the above studies tend to provide further support that an isolated anti-DFS70 result does makes a SARD diagnosis highly unlikely.

1.2.4 Clinical appropriateness of anti-DFS70

Determining whether or not the presence of anti-DFS70 antibodies can be used to eliminate a SARD diagnosis has become increasingly important, especially since ANA referral patterns have changed over recent years. The HEp-2 ANA test was originally usually only requested by Rheumatologists and Clinical Immunologists, however

requests are now being received from other specialties as well. This is because many other diseases with autoimmune features are also associated with ANAs (Mahler & Fritzler, 2012). However there is a danger that inappropriately testing for HEp-2 ANA outside the correct clinical setting can result in a high proportion of ANA positive patients that do not have SARD as this assay does have limited specificity (Meroni et al., 2014). This could then impact on the diagnosis and treatment of these patients. If the presence of anti-DFS70 antibodies could be used to eliminate a SARD diagnosis, then this may account for a significant portion of these 'false positive' results (Miyara et al., 2013).

Some of the anti-DFS70 studies performed in a general hospital laboratory have noticed differences in the frequency of anti-DFS70 amongst the different specialties sending the request. For example, Miyara et al. (2013) noted that in their study the prevalence of the DFS pattern in samples referred from internal medicine / rheumatology was lower than other IIF patterns, whereas the opposite was found in samples from neurology. The danger of inappropriate testing for ANAs was highlighted in a case where an 8-year-old girl had the typical clinical and laboratory findings of acute glomerulonephritis; however the clinical diagnosis could easily have been misinterpreted as an autoimmune condition as she also presented with a strong positive ANA result. Since she was positive for anti-DFS70 but negative for all other significant ANA antibodies, the authors concluded that the presence of isolated anti-DFS70 antibodies may be useful to exclude SARD in children as well (Fabris et al., 2014). Some of the anti-DFS70 studies performed in a general hospital laboratory do not state from which clinical specialties the ANA requests were received from. This would be worth noting as the prevalence of anti-DFS70 they reported may be affected by the proportion of rheumatology and non-rheumatology samples in the cohort.

The cause of the reduced prevalence of anti-DFS70 in SARD patients is unclear. It has been suggested that it may be due to the therapeutic treatments of these patients as they are usually on immune suppressants and corticosteroids (Miyara et al., 2013). Further investigations using newly diagnosed SARD patients not yet on any treatment would be required in order to substantiate this claim. It appears to be a significant limitation for most anti-DFS70 studies as most of these studies do not state whether the SARD patients in their cohort were newly diagnosed or not, nor whether they were on any immune suppressant drugs at the time of testing. For example, Miyara et al. (2013) did

not state whether or not the SARD patients in their study group had been previously diagnosed or if they were on any immune suppressing treatments at the time. They used known SARD positive patients as their control group, however they did not indicate if these patients were on any immune suppressing treatments at the time, although it is likely that most of them were on some form of treatment.

In addition, it is also not certain as to whether demographic, genetic or even environmental factors may be affecting the prevalence of anti-DFS70 (Mahler & Fritzler, 2012). In the Watanabe study the cohort consisted of Japanese subjects only and the authors' acknowledged that similar studies with other ethnic groups were required (Watanabe et al., 2004). Another study that used a Brazilian cohort also produced similar conclusions to Watanabe study which may indicate that race may not be a factor (Dellavance et al., 2005), however these observations do need further expanding with different ethnic groups.

As well as ethnicity, the prevalence of anti-DFS70 may also vary according to age and sex. Multiple studies have shown that anti-DFS70 antibodies are more prevalent among females and younger individuals (Watanabe et al., 2004, Muro et al., 2008 and Dellavance et al., 2005). Conversely, a study by Mahler et al. (2012) did not find a significant difference in the prevalence of anti-DFS70 according to age or sex. They suggested that the reason for this may be due to the differences in the cohorts used and so this indicates that this area also requires further investigation.

1.2.5 Challenges in the identification of anti-DFS70

In the ANA HEp-2 IIF assay, the DFS pattern typically appears as dense fine speckles uniformly distributed in the nucleus of interphase cells, whilst densely staining the chromatin region of the metaphase cells (Figure 1). Therefore it can be distinguished from the traditional speckled pattern which does not stain the metaphase chromatin (Bizzaro et al., 2015), although accurate identification of the DFS pattern has been shown to be quite challenging (Bizzaro, Tonutti, & Villalta, 2011). This may be why the prevalence of anti-DFS70 antibodies often varies significantly between studies. For example, Muro et al. (2008) reported a prevalence of 4% in SARD patients whereas Miyara et al. (2013) reported a prevalence of 13%. There are even significant differences in the reported frequencies of the DFS pattern in other disease groups as well in healthy individuals (Bizzaro et al., 2015). The DFS pattern can easily be

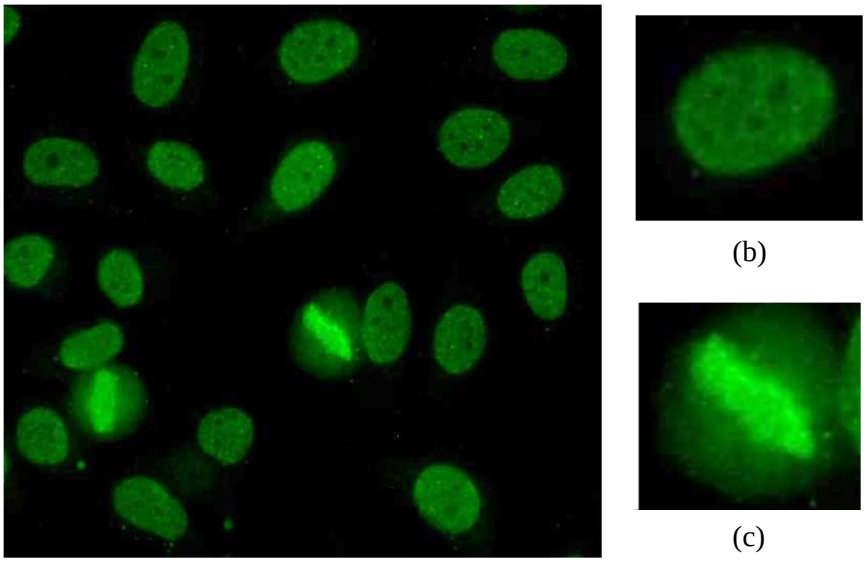


Figure 1. Indirect immunofluorescence (IIF) on HEp-2 cells depicting the typical dense fine speckled (DFS) pattern. (a) wide-angle view showing both interphase and metaphase cells, (b) dense fine speckles uniformly distributed in the nucleus of an interphase cell, (c) the chromatin region is densely stained in a metaphase cell.

confused with other patterns as it appears very similar to other speckled ANA patterns and it can sometimes be hard to distinguish between the DFS pattern and some low titer homogenous patterns. Mixed patterns may also mask its presence (Lee, Kim, Han & Oh, 2016).The difficulty in accurately identifying the DFS pattern was highlighted in a recent survey which assessed how accurately the DFS pattern was recognized by technologists with diverse levels of ANA IIF pattern recognition experience, ranging from less than a year to greater than ten years of experience. Results showed that only half the participants were able to recognize the DFS pattern and less than 10 % correctly identified mixed patterns from sera containing both anti-DFS70 and another clinically relevant ANA (Bentow, Fritzler, Mummert & Mahler, 2016a).

The limitations of the IIF ANA screen may also be contributing to the differences in reported frequencies of anti-DFS70 in SARD patients. As mentioned previously, whilst the IIF technique has been recommended for ANA screening by the American College of Rheumatology, this assay does have inherent limitations such as subjective interpretation of results and lack of standardisation of the assay across different laboratories (Mahler et al., 2014). These limitations have been known to be the cause of significant differences in the staining patterns seen between ANA IIF kits from different manufacturers (Copple et al., 2014). Previous studies have shown that the DFS pattern can be identified on slides from a number of different commercial ANA kits (Miyara et

al., 2013, Gundin et al., 2016, Mutlu, Eyigör, Mutlu, & Gültekin, 2016). In order to compare the staining pattern of anti-DFS70 positive samples, Bizzaro et al. (2011) tested the same anti-DFS70 positive samples on ANA kits from four different manufacturers. Of the thirteen anti-DFS70 positive samples tested, just three of these samples were positive on all four cell lines. When another group attempted a similar experiment, this time testing serum from an apparently healthy individual on slides from four different manufacturers, the DFS pattern was detected on each one, although they did appear slightly different (Mahler et al., 2012). This suggests that further studies are required in order conclude that the use of different substrates may contribute to different results. This lack of inter-laboratory standardisation is also a severe limitation of large studies that have used samples from multiple laboratories. This is the case of the Bizzaro et al. (2015) study where samples from several different laboratories where used. In this study each laboratory had originally identified the DFS pattern using their own ANA IIF kits which were from different manufacturers and then forwarded the samples to the group for anti-DFS70 testing. The group detected anti-DFS70 in 30% of the samples presenting a DFS pattern, so this figure may have been different if all the laboratories in the study had used the same ANA HEp-2 IIF kit.

The challenges presented above in correctly identifying the DFS pattern supports a general consensus that the presence of anti-DFS70 should not be reported if identified by ANA IIF alone. Instead, its presence should be confirmed by using an assay specifically designed to detect it and the result should be incorporated into ANA test algorithms. Anti-DFS70 antibodies can now be specifically tested for by ELISA, immunoadsorption IIF, immunoblot and CIA (Lee et al., 2016). Some studies have not confirmed the presence of anti-DFS70 and so their results should be interpreted with caution. For example, in a study conducted by Pazini, Fleck, dos Santos & Beck (2010) their aim was to determine the frequency of the DFS IIF pattern with a possible clinical correlation with SARD in their study population. However they did not confirm the presence of anti-DFS70 on those samples showing the DFS pattern and doing so would have presented superior results.

While there is a growing consensus to include the anti-DFS70 result into ANA test algorithms, there has been no clear protocol agreement on when to test for it and how to incorporate it into the algorithm. In addition, anti-DFS70 antibodies have been identified in other IIF patterns as well (Gundin et al., 2016), which suggests that all

ANA positive results should be tested for anti-DFS70 antibodies and not just those exhibiting a DFS pattern alone. This has not been the case in most studies to date. For example, the studies mentioned above (Mahler et al., 2012, Miyara et al., 2013 and Mutlu et al., 2016) all tested for anti-DFS70 on samples that had shown a DFS pattern only. As mentioned previously, the DFS pattern may be difficult to recognize therefore it is highly likely that some anti-DFS70 positive patients may have been missed in these studies. Consequently the prevalence of anti-DFS70 may actually be higher than reported. Gundin et al. (2016) recommended that testing for anti-DFS70 antibodies should only be performed on those samples that are ANA IIF positive, but negative for all other clinically relevant ANAs.

1.2.6 Anti-DFS70 literature

It is worth noting that the majority of the anti-DFS70 published literature is authored or co-authored by at least one individual with links to Inova Diagnostics Inc. For example M. Mahler and C. Bentow are both employees of Inova Diagnostics Inc., M.J. Fritzler is a consultant of theirs and Dr. Bizzaro has received consultant fees from the Werfen Company, which is the parent company of Inova Diagnostics Inc. Therefore it appears that much of the research into anti-DFS70 has been driven by Inova Diagnostics Inc. Inova Diagnostics Inc. is a biotechnology company that develops, manufactures and sells autoimmune technologies and diagnostic markers world-wide. They have a CIA test (CLIA-QUANTA-Flash DFS70) for anti-DFS70 detection and they have also developed an immunoadsorption assay (HEp-2 Select, Inova) that assists in anti-DFS70 detection. Nevertheless, these authors have declared their conflict of interest in each case and in most instances they have co-authored with other individuals that have declared no conflict of interest. It is also reassuring to note that researchers with no links to Inova Diagnostics Inc. are producing similar results and coming to similar conclusions with regards to anti-DFS70. For example, Lee, Kim, Han, & Oh (2016) concluded that the anti-DFS70 result should be included in the ANA test algorithm as it can improve the efficiency in diagnosing SARD.

1.3 Current ANA Testing in New Zealand

At the initial planning stages of this study, no New Zealand public or private clinical laboratories were routinely testing for anti-DFS70. Additionally, no studies or evaluations on anti-DFS70 had been performed in a New Zealand clinical laboratory,

although there was an interest in determining if specifically testing for anti-DFS70 would aid in the diagnosis of SARD in a New Zealand population. This is because there could be a high possibility that patient results would get mistakenly reported as a different pattern such as speckled or a mixed homogenous / speckled pattern which could impact on the diagnosis and management of these patients. Thus the proposed aim was worth investigating as there was a need to determine if the prevalence of anti-DFS70 in New Zealand is significant enough to warrant identification and further investigation. Also, New Zealand and in particular Auckland, is ethnically diverse and as mentioned previously, most studies have generally been limited a single ethnic group and it is unknown as to whether genetics or geographic location can influence the prevalence of anti-DFS70.

To date, there have been no reported studies on the prevalence of anti-DFS70 in the New Zealand population, although now some New Zealand diagnostic laboratories have performed evaluations with regards to anti-DFS70. Currently one New Zealand laboratory specifically tests for anti-DFS70 and has included its result in their ANA diagnostic algorithm. Others have performed or are in the process of performing their own evaluations.

1.4 Aims and Objectives

The purpose of this research is to determine whether or not New Zealand diagnostic laboratories should be specifically testing for the presence of anti-DFS70 antibodies and reporting the result to the clinicians. In order to achieve this, the principal objectives are: (1) to determine if routine ANA testing methods used in a New Zealand diagnostic laboratory are detecting anti-DFS70 antibodies, (2) to determine the local prevalence of anti-DFS70 in ANA positive patients in a New Zealand public hospital population, (3) to determine if the presence of anti-DFS70 is clinically significant in terms of a SARD diagnosis. Should anti-DFS70 antibodies prove to be a significant factor in terms of eliminating a SARD diagnosis, then a new ANA diagnostic algorithm may be proposed.

The research questions for each objective are listed as follows:

Objective 1: To determine if routine ANA testing methods used in a New Zealand diagnostic laboratory are detecting anti-DFS70 antibodies. The research questions are:

(1) Is anti-DFS70 detected by standard IIF and ELISA techniques? (2) If so, which is better at detecting anti-DFS70; IIF or ELISA? (3) Is an automated IIF slide reader capable of flagging the DFS IIF pattern? (4) Is anti-DFS70 present in other IIF patterns?

Objective 2: To determine the local prevalence of anti-DFS70 in ANA positive patients in a New Zealand public hospital population. The research questions are (1) What is the local prevalence of anti-DFS70 in ANA positive patients suspected of having SARD and those with known SARD? (2) Is there a significant difference in the prevalence of anti-DFS70 according to age, sex and ethnicity? (3) Are the ANA referral patterns an influencing factor of the prevalence of anti-DFS70 in a public hospital?

Objective 3: To determine if the presence of anti-DFS70 is clinically significant in terms of a SARD diagnosis. The research questions are (1) Is the presence of anti-DFS70 (found alone or with other ANA's) clinically significant in terms of a SARD diagnosis? (2) Is the amount of anti-DFS70 detected clinically significant in terms of a SARD diagnosis? (3) How should the anti-DFS70 result be incorporated into the ANA test algorithm?

2 Methodology and Methods

2.1 Methodology

2.1.1 ANA screen

As mentioned previously, the ANA HEp-2 IIF assay is the Gold Standard assay for ANA detection and is the method of choice for ANA detection as recommended by the American College of Rheumatology (American College of Rheumatology, 2009). However due to the known subjective and technical limitations of this assay, there have consequently been attempts to replace this assay with more economical and higher throughput immunoassays such as bead-based multiplex platforms and other solid phase assays such as ELISA. These assays test for most of the key SARD autoantibodies in a single run. Since they are fully automated they are faster, cheaper, easier to perform and they also eliminate all subjective interpretation (Mahler & Fritzler, 2012). However a limitation of these assays is that they do not produce an ANA pattern or titre, which clinicians may find useful for SARD diagnosis and management. Nevertheless, these alternate methods could still be used, with any positive results followed by ANA HEp-2 testing to confirm positivity and determine the titer and fluorescence pattern. Thus this would optimise work flow and reduce costs and turnaround times, particularly in larger laboratories (de Almeida Brito, et al. 2016). In the context of anti-DFS70, Mariz et al. (2011) have shown that the DFS pattern is not only found in low titers, but in higher titers as well. In fact, most of the samples with the DFS pattern in their study had a high ANA titer and it was even seen in titers greater than 1:5120. Therefore whilst they concluded that the IIF titer and patterns do help to discriminate between ANA positive healthy individuals and patients with SARD, they also concluded that the ANA titer is not important when the DFS pattern is present.

Another limitation of these newer assays is that since they do not contain the full repertoire of ANA autoantigens, they appear to have a high prevalence of ‘false negatives’ (Aggarwal, 2014). Although studies have shown that in some cases the sensitivity of these alternative assays are comparable to or even higher than the sensitivity of ANA IIF assay. There is also a lack of standardisation among these assays regarding their antigenic composition and variation in cut-off levels thus they do have highly variable sensitivities and specificities between them (de Almeida Brito et al.,

2016). For this reason there has been a recent recommendation by an international expert panel that the ANA IIF assay be the preferred method for ANA detection, however they do acknowledge the shortcomings of the IIF assay and have allowed for the use of alternative assays. In the case where an alternate method has been used but the result is negative when the clinical suspicion of SARD is high, they recommend that the ANA IIF assay should be performed in order to confirm the result (Agmon-Levin et al., 2014). This international panel as well as the American College of Rheumatology also recommend that laboratories should specify which ANA detection method was used in the result report (American College of Rheumatology, 2009).

A review of the literature shows that it is not clear as to whether these newer ANA screening assays are also detecting anti-DFS70 antibodies. One study suggests that ELISA may not be detecting anti-DFS70 antibodies as only 35% of the samples in their study that showed the DFS pattern were positive on the ELISA ANA screen (Miyara et al., 2013). It appears that this area requires further investigation, perhaps using ANA ELISA kits from a number of different manufacturers. Miyara et al. (2013) also tried to determine if the ANA ELISA result could be combined with the DFS70 CIA result in order to give a diagnostic score which would provide further help in distinguishing between SARD and non-SARD ANA IIF positive patients. They found that it did appear to significantly improve discrimination between SARD patients and non-SARD patients. Further research into this area has not been performed since, however it appears that it may be worth exploring.

Due to the limitations inherent in both the ANA IIF and ANA ELISA assays, in order to increase the likelihood of identifying anti-DFS70 positive patients in this study, patient samples were tested for ANAs using an ANA IIF assay as well as an ANA ELISA assay. This then allowed for comparison between the two assays in order to determine which is better able to detect anti-DFS70 antibodies. An ELISA/CIA diagnostic score was not included in this study and since there is evidence that ANA titers are not important when the DFS pattern is present, ANA titers were also not considered for this study's purposes.

2.1.2 Automated ANA IIF slide readers

The American College of Rheumatology recommendation to return to ANA testing by HEp-2 IIF generated a need to eliminate some of the limitations in the IIF test system.

This resulted in the development of automated ANA IIF slide readers which aimed to standardise slide reading and thus remove some of the subjectivity of the assay. In this way intra and inter laboratory variability could be significantly reduced. Other advantages include a higher throughput as slides can be read at a faster rate, a darkroom is also no longer required and images can be stored and retained for many years. These images can also be easily forwarded to specialists should further advice on interpretation be required (Meroni et al., 2014). The IIF slide readers work by using fluorescent intensities with predefined cut-off values for positive/negative reporting and then mathematical pattern recognition software is used to interpret the most common patterns. Operators can then agree with the reader or have the option of changing the result if needed (Copple et al., 2014). Some IIF automated slide readers have an extra function in that they have a quantitative fluorescence intensity value which is equivalent to the end-point titer. This therefore eliminates the need to do end point titers on all positive samples, which not only saves time but also reduces costs (Meroni et al., 2014).

To date none of the automated IIF slide readers are capable of recognizing the DFS pattern (Bizzaro et al., 2015). In a recent study Bizzaro et al. (2013) investigated the pattern recognition accuracy of seven different automated IIF slide readers. Whilst they all were able to identify the DFS positive samples as ANA positive, the patterns were incorrectly classified as either homogenous or speckled. It is important that automated slide readers do not incorrectly classify the DFS pattern in the first instance as it could lead to incorrect reporting of ANA patterns to the clinicians. Further research into this area is required.

In order to reduce subjective interpretation in this study, all ANA HEp-2 IIF slides were initially read by an IIF automated slide reader called the NOVA View®, with results confirmed or changed by the operator. Thus it could also be determined whether or not the NOVA View was capable of flagging the DFS IIF pattern.

2.1.3 Anti-DFS70 detection

Whilst there are currently various methods for anti-DFS70 detection such as ELISA, immunoadsorption IIF, immunoblot and CIA (Lee et al., 2016), it was decided that this study would use the QUANTA Flash® DFS70 CIA (Inova Diagnostics). The main reason for this was because most of the previous research had used this assay for anti-DFS70 detection. For example, Bizzaro et al. (2015), Mahler et al. (2012) and Miyara et

al. (2013) all used the QUANTA Flash® DFS70 assay. Another method would likely have differences in specificity and sensitivity; therefore by using the same assay as most previous studies, the resulting prevalances obtained in this study could be better compared with that of previous studies. Also, the laboratory where this study was performed had a BIO-FLASH instrument available for use, which is the platform that the QUANTA Flash DFS70 assay runs on. Thus the kit was also easily sourced from the supplier.

2.1.4 Samples size and requirements

Samples used were clinical samples sent to a public hospital laboratory for routine ANA testing. The total number of samples tested was limited due to budget constraints. It was decided that a minimum of 100 ANA positive samples each from SARD and non-SARD patients would be used in this study as this would be a sufficient number of samples to determine the difference in prevalence between these two groups in order to satisfy Objective 2. Samples collected would be a consecutive series of samples until 100 of each group was obtained. By using consecutive samples, this would also provide a random selection of ethnicities, ages and sex in the hope that there would be sufficient representation of each demographic group and sub group in the study cohort in order to determine if the prevalence of anti-DFS70 differs according to each of these factors.

Requests for ANA testing were sent from different specialties and not from the Rheumatology department alone, therefore the patients had a variety of conditions. Clinicians had requested ANA testing on these patients as they had either suspected the patient had an autoimmune condition, for the differential diagnosis from SARD or for treatment monitoring of known SARD patients. Patients were classified as having SARD if they were first time ANA positive with symptoms strongly suggestive of a SARD condition or were previously diagnosed with one or more of the following conditions: Rheumatoid arthritis (RA), SLE, Sjögren's Syndrome (SjS), Systemic Sclerosis (SSc), and polymyositis (PM) / dermatomyositis (DM).

2.1.5 Quality assurance

Two Royal College of Pathologists of Australia Quality Assurance Programme (RCPAQAP) specimens known to be positive for anti-DFS70 were also analysed in order to confirm whether or not anti-DFS70 was being detected by both the ANA assays and by the anti-DFS70 CIA assay used in this study. The RCPAQAP provides external

quality assurance (EQA) for clinical laboratories worldwide. Their ANA EQA program involves monthly testing of EQA samples for ANA by IIF or any other alternative method used by the laboratory. Pattern interpretation and titer are also performed. Results are collated and returned to the laboratories so they can track how well their ANA testing process is performing compared with other laboratories.

2.1.6 Ethics

This study was given full ethical approval by the New Zealand Health and Disability Ethics Committee (HDEC) on 15 July 2015. Reference number 15/CEN/103 (Appendix A).

Since patient blood samples were used the key ethical considerations is to ensure patient confidentiality and that the samples were used appropriately. All research was carried out at North Shore Hospital which is part of the Waitemata District Health Board (WDHB) and within the WDHB there are policies and guidelines in place that comply with the obligations under the relevant legislations including the Human Tissue Act 2008, Treaty of Waitangi Act 1975 and Health and Disability Commissioner, 1996. These policies include ensuring patient confidentiality with appropriate handling of patient samples and accessing of clinical details. Full ethical approval was also given by the WDHB Ethics Committee as well as the WDHB Maori Ethics Committee (Appendix A).

The blood samples used in this study were those that had been analysed for ANA testing. The patients' clinical details required for this study include ethnicity, sex, age, diagnosis and previous ANA results and these were found by accessing the patients' medical records.

2.2 Methods

All samples sent for routine ANA testing to the laboratory were tested by two ANA detection methods (HEp-2 IIF and ELISA) in parallel. For the purposes of this study, a sample was considered ANA positive if it was positive by at least one of these methods. The HEp-2 IIF slides were initially read by an automated IIF slide reader and checked by the operator who accepted or changed the pattern interpretation. All ANA positive samples were then tested for ENA antibodies and anti-dsDNA by ELISA as part of the

standard testing process in the laboratory. Once tested, these samples were then stored frozen and batch tested for anti-DFS70 by CIA at a later date. A consecutive series of routine ANA positive samples were collected until 100 each of ANA positive SARD and non-SARD samples were obtained. Once all testing was completed, patient demographics and clinical details were obtained from patient clinical records and results were analysed. Figure 2 summarises this study process.

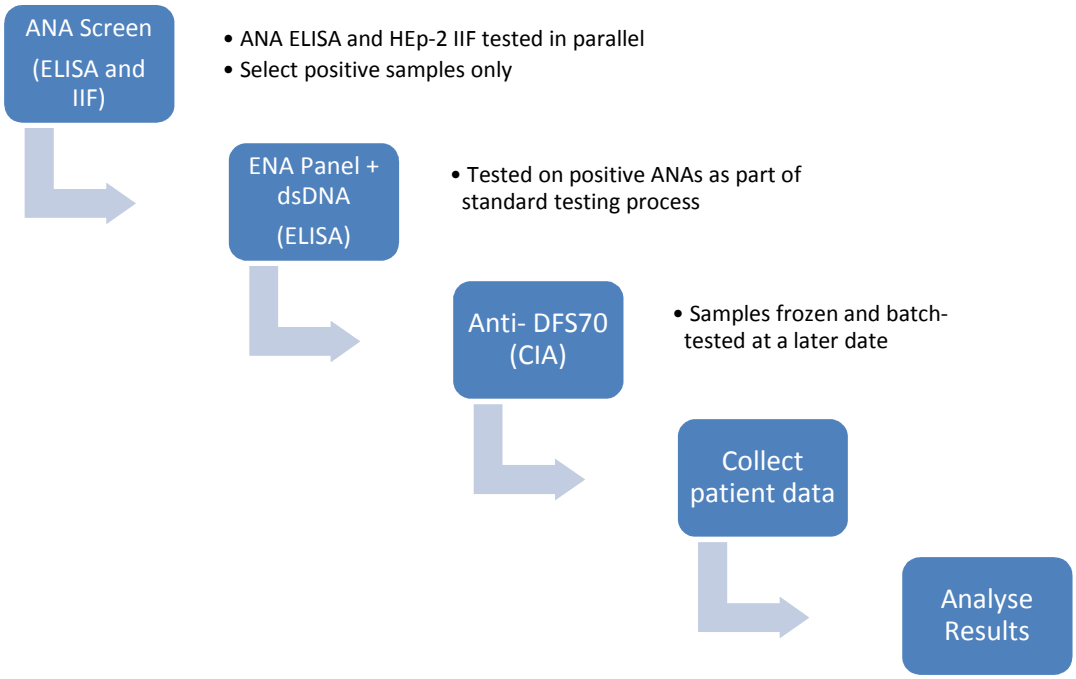


Figure 2. Conceptual overview of the study design.

2.2.1 Sample collection and storage

Patient blood was collected into serum separating tubes (SST). Samples were spun at 3000 revolutions per minute (rpm) for 10 minutes and serum was separated and stored at 4°C until ANA testing was performed. All serum samples were initially tested for ANAs by ELISA and IIF. ANA positive samples were subsequently tested for specific ENA antibodies (anti-SSA, anti-SSB, anti-Sm, anti-Sm/RNP, anti-Scl-70, anti-dsDNA and anti-centromere). These samples were then stored frozen at -30°C until sufficient samples had been collected for batch testing of anti-DFS70 testing by CIA.

2.2.2 ANA testing

All samples were tested for the presence of ANAs by two ANA detection methods; namely an IIF assay and an ELISA assay. For the purposes of this study a sample was considered ANA positive if it had a positive result for either of these assays.

2.2.2.1 IIF ANA screen

The NOVA Lite® HEp-2 ANA kit with DAPI (INOVA Diagnostics) was used for the IIF method. All samples were processed manually according to the manufacturer's protocol (INOVA Diagnostics, 2012) at a dilution of 1:80. Reading and interpretation of immunofluorescent patterns were performed by the NOVA View® slide reader, with software version 1.04 according to the manufacturer's protocol (INOVA Diagnostics, 2014). The NOVA View slide reader contains an Olympus IX81 inverted fluorescence microscope with dual band DAPI FITC/HC filters. Digital images are captured by a Kappa DX4 digital camera attached to the microscope. Determination of whether a sample is positive or negative is defined by a pre-set cut-off of Light Intensity Units (LIU) and for ANA HEp-2 slides the LIU cut-off is set at 48 LIU. The NOVA View classified the IIF patterns as speckled, homogeneous, centromere, cytoplasmic, nucleolar or unrecognised (INOVA Diagnostics, 2014). Results were checked and confirmed by the operator or changed if the operator did not agree with the NOVA View interpretation. For the purposes of this study, all ANA IIF patterns were classified as speckled, homogeneous, centromere, cytoplasmic, nucleolar or other. The NOVA View is not capable of recognising the DFS pattern however it was defined by the operator as speckled staining of the nucleus with positive mitotic staining.

2.2.2.2 ELISA ANA screen

The Autoimmune enzyme immunoassay (EIA) ANA Screening Test (Bio-Rad) was used for the ELISA method. This is a qualitative assay which is intended to screen for the presence of ANAs in human serum. In a single well, this assay collectively detects total ANAs against double stranded DNA (dsDNA, nDNA), histones, SS-A/Ro, SS-B/La, Sm, Sm/RNP, Scl-70, Jo-1, and centromeric antigens, as well as sera positive for IIF HEp-2 ANAs (Bio-Rad, 2011). The assay was run on the fully automated EVOLIS™ System (Bio-Rad) and performed according to the manufacturer's protocol. Samples with a calculated ANA number (optical density (OD) of test sample/OD of cut-off) greater than or equal to 1.0 were considered positive, as suggested by the manufacturer (Bio-Rad, 2011a).

2.2.3 ENA panel

Detection of specific ANAs was performed on all ANA positive samples by ELISA on the EVOLIS™ System (Bio-Rad) according to the manufacturer's protocol.

The Autoimmune EIA ANA-6 Profile Test (Bio-Rad) was used for ENA antibody detection. This assay is used for the semi-quantitative measurement of anti-SSA/Ro, anti-SSB/La, anti-Sm, anti-SmRNP, anti-Scl70 and anti-Jo-1. Results are calculated in Enzyme Units (EUs), with positive samples having an EU of greater than 25EU, as recommended by the manufacturer (Bio-Rad, 2010).

The Autoimmune EIA anti-dsDNA Test (Bio-Rad) was used for anti-dsDNA antibody detection. This is a quantitative assay intended to screen for the presence of dsDNA antibodies in human serum. Results are measured in International Units (IUs) with positive samples having an IU of greater than or equal to 25IU, as recommended by the manufacturer (Bio-Rad, 2011b).

The Autoimmune EIA anti-Centromere Test (Bio-Rad) was used for anti-centromere antibody detection. This is a semi-quantitative assay for the detection of IgG autoantibodies against the centromere antigen in human serum. Results are measured in EUs with positive samples having an EU of greater than 25EU, as recommended by the manufacturer (Bio-Rad, 2006).

2.2.4 Anti-DFS70 detection

All ANA positive patient samples were tested for the presence of anti-DFS70 by CIA using the QUANTA Flash® DFS70 assay (INOVA Diagnostics) on the automated BIO-FLASH® analyser (Biokit S.A) according to the manufacturer's protocol. This is a semi-quantitative assay. The principle of the method is based on using recombinant DFS70 antigen coated onto paramagnetic beads. In a positive reaction, anti-DFS70 antibodies will bind to the DFS70 antigen on the beads. Isoluminol conjugate bound to antihuman IgG is then added and this binds to the anti-DFS70. Once the conjugate is activated, relative light units (RLUs) are produced and measured, and these are proportional to the amount of anti-DFS70 present. Then using a standard curve, RLU values are converted into chemiluminescent units (CU). Samples with a CU of greater than or equal to 20 were considered to be positive, as recommended by the manufacturer (INOVA Diagnostics, 2013). The QUANTA Flash® DFS70 assay is also the current RCPAQAP reference method for anti-DFS70 detection.

2.2.5 Results analysis and statistical evaluation

Once all testing was completed, patient clinical details, demographics (age, sex and ethnicity) and the referring department were determined by accessing medical records.

The patient demographics were then analysed by ethnic group, sex and age group. Patient ethnicities were classified into seven broad ethnic groups. These were NZ European (Europeans born in New Zealand), Other European (British, South African, Italian, Russian, Australian), Maori, Asian (Chinese, Japanese, Indian, Korean, Philippino, Malaysian), Middle Eastern (Iran, Iraq), Pacific Islander (Tongan, Fijian, Samoan), and Other (Croatian, Latin American, African). As there was not enough samples represented in each age decade, for statistical purposes patient ages were classified into 4 age groups (10-40 years, 40-60 years, 60-80 years and greater than 80 years). Ethnic group, sex and age group were then further broken down into SARD status so that the prevalence of anti-DFS70 amongst each of these groups and sub-groups could be determined. The prevalence of anti-DFS70 in the SARD and non-SARD patients was also determined. The difference in anti-DFS70 prevalence amongst these variables was analysed by Fisher's exact test as the sample size was relatively small. For all statistical tests *P* values less than 0.05 were considered significant. Data was statistically evaluated using SAS® software (Version 9.4, 2016).

The anti-DFS70 positive patients' clinical diagnosis and symptoms were compared in order to determine if there were any commonalities amongst these patients and also to determine if they were clinically significant in terms of a SARD diagnosis. Anti-DFS70 seen in isolation was compared with those seen in conjunction with ENAs in order to determine if this was clinically significant in terms of a SARD diagnosis. The referring specialty for each of the anti-DFS70 positive samples was also noted and analysed for possible common referral patterns for these patients and to determine if ANA referral patterns are an influencing factor on the prevalence of anti-DFS70 found in this study.

The ANA detection rate between the IIF and ELISA assays was compared and then the detection rate of anti-DFS70 was compared between the two assays. The values obtained for each assay (LIU for the IIF assay and ANA# for the ELISA assay) were assessed in order to explain any discrepancies. The prevalence of anti-DFS70 for each of the main IIF patterns (homogenous, speckled, centromere) was then determined.

The amount of anti-DFS70 detected by CIA was used to determine if there was any correlation between the strength of the anti-DFS70 positivity and a SARD diagnosis. This was achieved by comparing the average amount of anti-DFS70 (in CU) in the non-SARD patients to the average amount found in SARD patients.

3 Results Analysis

3.1 Clinical Samples

A total of 211 ANA positive patient samples were used in this study (163 females, 48 males, mean age 53, age range 13-98). Of these, 102 were known SARD patients (86 females, 16 males, mean age 45, age range 18-79) and 109 were non-SARD patients (77 females, 32 males, mean age 60, range 13-98). The breakdown of SARD patients were as follows: Rheumatoid arthritis (RA) (n = 10), SLE (n = 73), Sjögren's Syndrome (SjS) (n = 24), Systemic Sclerosis (SSc) (n = 3) and polymyositis (PM) / dermatomyositis (DM) (n = 1). These numbers do not add up to the total number of SARD patients as some patients had more than one condition. (See Appendix B for entire research output).

3.2 Clinical Association of anti-DFS70 Antibodies

In a total of 211 patients, 109 non-SARD and 102 SARD ANA positive serum samples were tested for anti-DFS70 by the CIA QUANTA Flash DFS70 method. Anti-DFS70 was present in 7/109 (7%) of the non-SARD ANA positive patients and in 1/102 (0.98%) of the SARD ANA positive patients (Appendix B). The prevalence of anti-DFS70 was significantly higher in non-SARD patients compared to SARD patients ($p = 0.0401$). Table 3 summarises the test results and clinical details for the anti-DFS70 positive patients. The non-SARD anti-DFS70 positive patients had a variety of symptoms and conditions with no obvious commonalities. The single SARD patient positive for anti-DFS70 (Sample 8) was a known SLE patient.

3.3 Detection of anti-DFS70 and Specific ANAs

Specific ANAs were tested for by ELISA in order to investigate the simultaneous presence of autoantibodies for SARD in patients with anti-DFS70 antibodies (Table 3, 'ENA panel results' column). Anti-DFS70 was the sole ANA present in 5/8 (62.5%) samples. Other specific ANAs detected were antibodies to SSA and Scl70 as well as anti-centromere antibodies; however none of these patients presented with a SARD condition. The SARD patient had no other specific ANAs present nor did they have a history of specific ANAs. SLE diagnosis would have been based on other criteria.

Table 3.

Test results and clinical details of anti-DFS70 positive patients.

Sample	ANA IIF pattern NOVA View /LIU	ANA IIF pattern operator	ANA ELISA / ANA #	ENA panel / EU + dsDNA /IU	Anti-DFS70 CIA / CU	Clinical details and ANA test history
1	Centromere 232	Centromere / homogenous	Positive 5.21	Anti-centromere 82	33	Non-SARD. Lung cancer, pulmonary sarcoidosis. History of anti-centromere antibodies.
2	Unrecognised 438	Speckled	Positive 2.75	Anti-Scl70 33	>450	Non-SARD. Recurrent blistering. Previous ANA positive (homogenous) and anti-Scl70.
3	Unrecognised 179	Homogenous	Positive 2.54	Negative	76	Non-SARD. Ulcerative colitis. No ANA test history.
4	Centromere 305	Centromere	Positive 2.91	Anti-SSA 29 anti-centromere 38	262	Non-SARD. Sudden loss of sensation, facial nerve distribution. Otherwise fit and well. No ANA test history.
5	Negative 24	Negative	Positive 1.06	Negative	119	Non-SARD. Primary Raynaud's, eosinophilic oesophagitis. Mild dry eyes, dry mouth – lupus investigations. Previous ANA positive (homogenous), ENA negative.

Table 3 *(continued)*

Sample	ANA IIF pattern NOVA View /LIU	ANA IIF pattern operator	ANA ELISA / ANA #	ENA panel / EU + dsDNA /IU	Anti-DFS70 CIA / CU	Clinical details and ANA test history
6	Homogenous 215	Homogenous	Positive 3.5	Negative[a]	52	Non-SARD. Epilepsy, two miscarriages. Positive anti-cardiolipin, positive lupus anticoagulant. Previous ANA positive (speckled), ENA negative.
7	Unrecognised 146	DFS	Positive 1.01	Negative	414	Non-SARD. Epigastric pain. No ANA test history.
8	Homogenous 107	Homogenous	Positive 1.98	Negative	137	SARD. SLE monitoring. Previous ANA positive (homogenous), ENA negative.

Note. ANA = antinuclear antibody; IIF = indirect immunofluorescence; ENA = extractable nuclear antigen; LIU = light intensity units; DFS = dense fine speckled; CIA = chemiluminescence immunoassay; CU = chemiluminescent units; EU = enzyme units; SARD = systemic autoimmune rheumatic disease; SLE = systemic lupus erythematosus. LIU cut-off = 48; ELISA ANA# cut-off = 1.0; ENA panel cut-off = 25EU; dsDNA cut-off = 25IU; anti-DFS70 cut-off = 20CU.

[a] ELISA dsDNA >200IU but probable false positive as was negative by Farr assay and patient clinical details did not indicate SLE.

Sample 6 was strongly positive for anti-dsDNA by ELISA. However since the patient's symptoms did not indicate SLE, further investigations revealed a negative result for anti-dsDNA using the Farr radioimmunoassay. It was concluded that the anti-dsDNA ELISA result was likely a false positive and therefore the anti-dsDNA result for sample 6 was considered negative for this study.

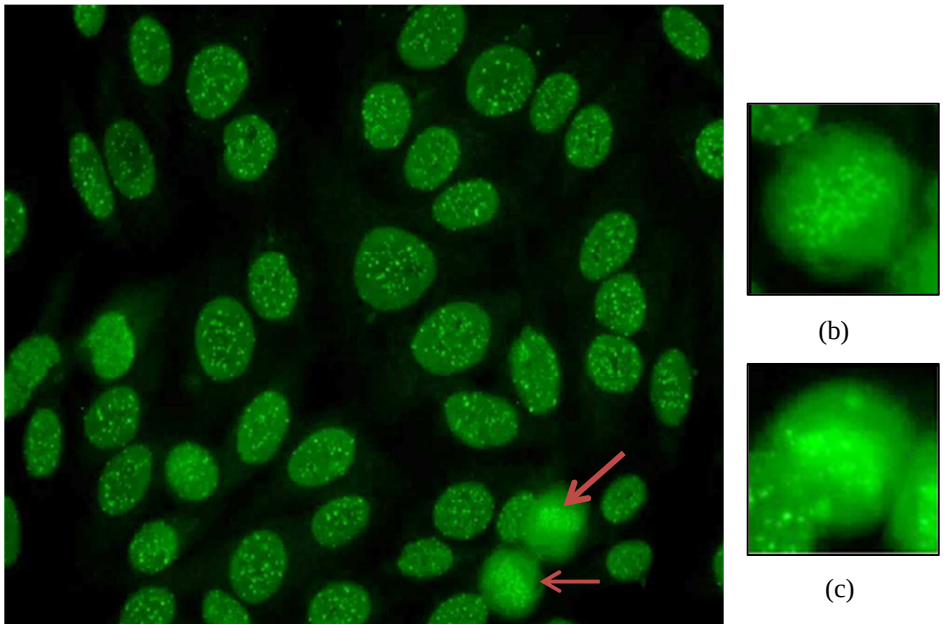
The amount of anti-DFS70 in the positive patient samples ranged from 33CU to greater than 450CU (mean=193CU). The SARD patient had a result of 137CU which was only slightly below the mean. This result indicates that there is no correlation between the strength of the anti-DFS70 positivity and a SARD diagnosis.

3.4 Agreement Between ANA ELISA and IIF

A sample was considered ANA positive if it was positive by either the ANA ELISA or IIF HEp-2 assay. Of the 211 ANA positive results, 8 samples were ELISA negative / IIF positive and 24 samples were ELISA positive / IIF negative. The ELISA ANA Screen was positive for all 8 anti-DFS70 positive samples, whereas the IIF ANA test was positive for 7/8 samples. However two of these eight ELISA ANA Screen results were just above the cut-off for positivity (ANA# >= 1.0) and this included the sample that was negative by IIF. According to the NOVA View, the 7 anti-DFS70 positive samples that were ANA positive all had LIUs well above the cut-off for positivity (48LIU). The negative sample was clearly negative at 24LIU (Table 3).

3.5 ANA IIF Pattern Interpretations

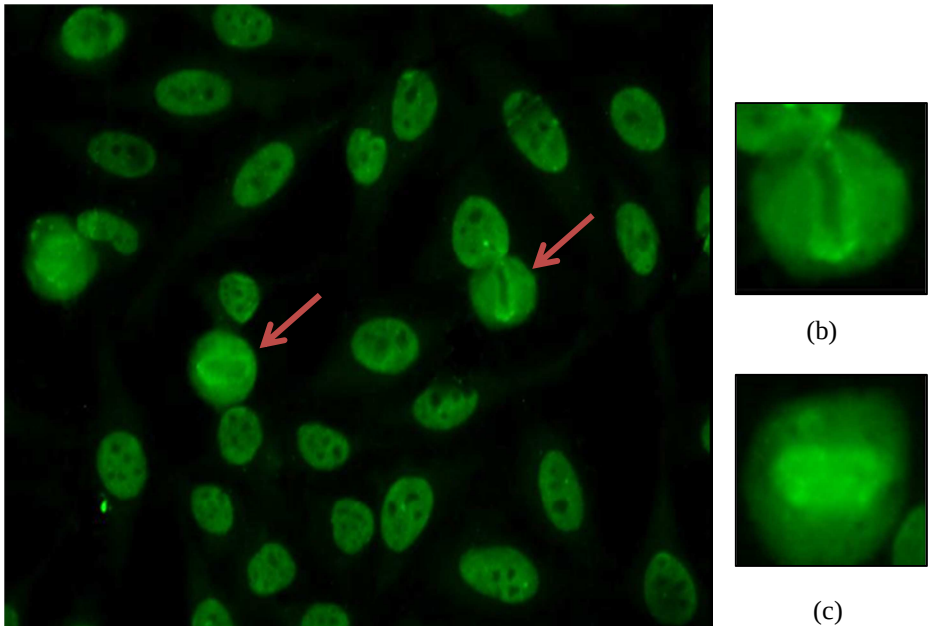
For sample 1 (Figure 3), the NOVA View classified this pattern as centromere only, however the operator disagreed and changed the pattern to a mixed centromere / homogenous pattern.



(a) (b) (c)

Figure 3. Sample 1 HEp-2 Indirect Immunofluorescence (IIF) images (a) wide-angle view showing both interphase and metaphase cells, (b) and (c) enlarged metaphase cells (as indicated by the red arrows).

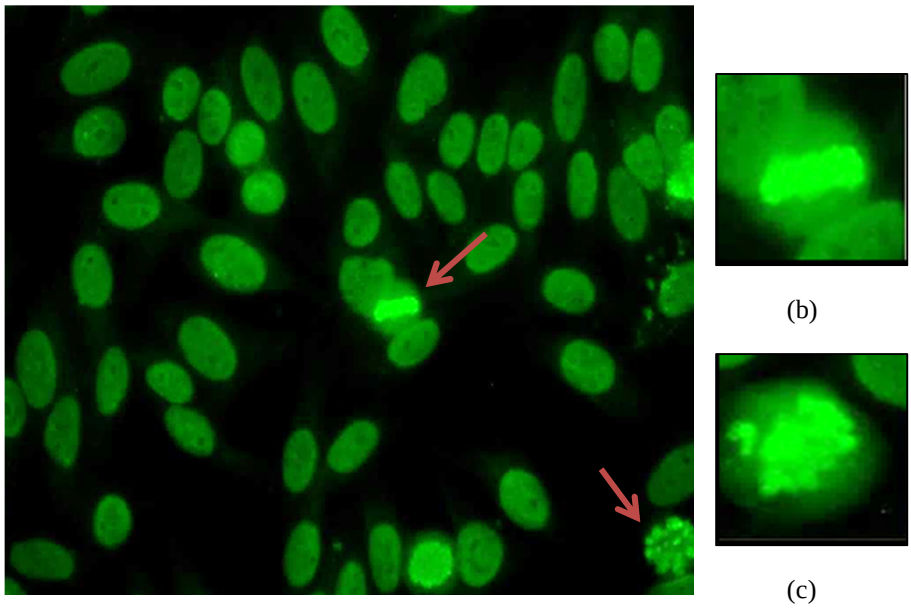
For sample 2 (Figure 4), the NOVA View was unable to classify this pattern, probably due to the mitotic cell on the right appearing to have positive staining and the one on the left appearing to have negative staining. The operator decided that this was a speckled pattern.



(a) (b) (c)

Figure 4. Sample 2 HEp-2 Indirect Immunofluorescence (IIF) images, (a) wide-angle view showing both interphase and metaphase cells, (b) and (c) enlarged metaphase cells (as indicated by the red arrows).

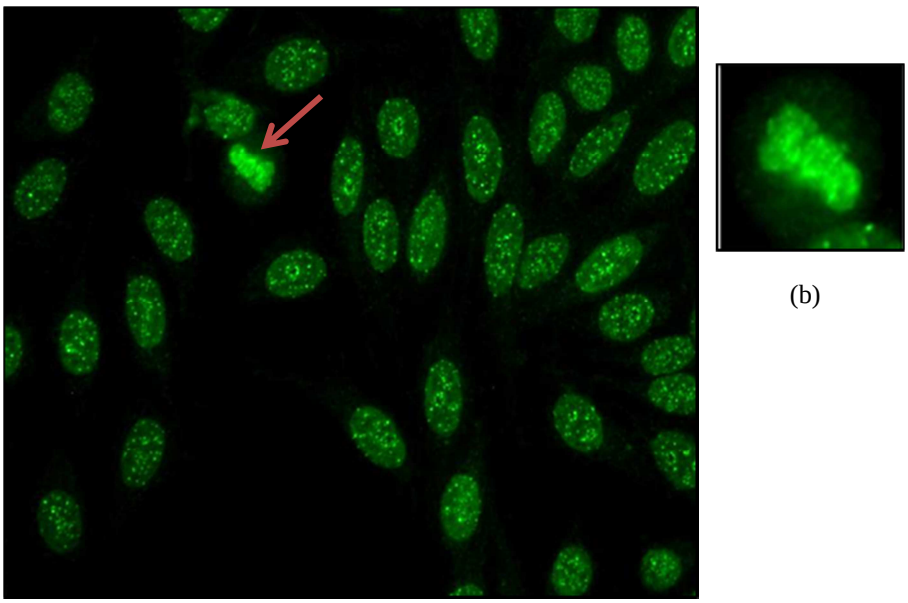
For sample 3 (Figure 5), the NOVA View was unable to classify this pattern, probably because it thought all three of the enlarged cells were mitotic cells. Due to the clear positive staining on the mitotic cell on the far left, the operator classified this pattern as homogenous.



(a) (b) (c)

Figure 5. Sample 3 HEp-2 Indirect Immunofluorescence (IIF) images, (a) wide-angle view showing both interphase and metaphase cells, (b) and (c) enlarged metaphase cells (as indicated by the red arrows).

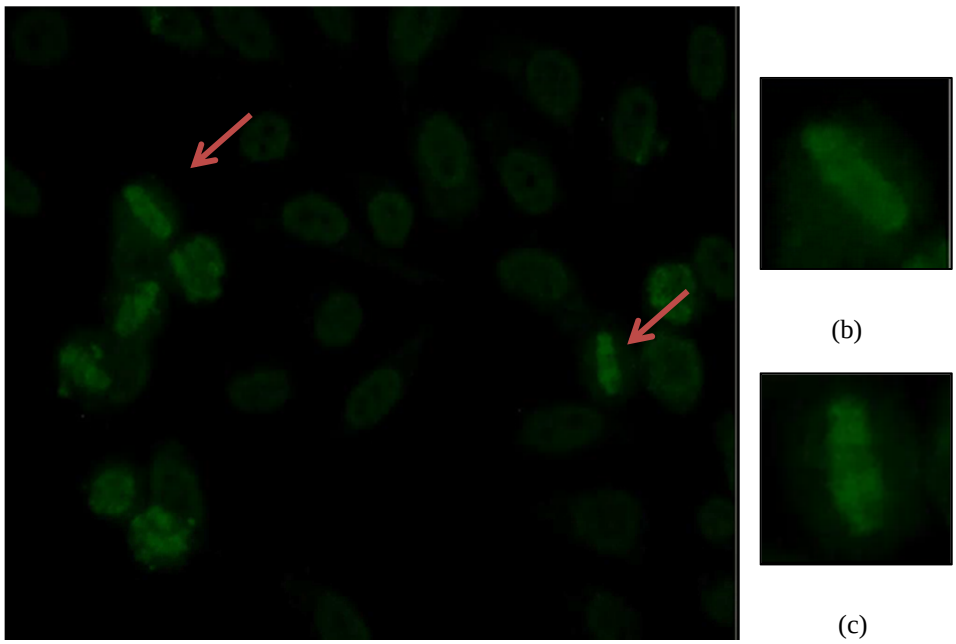
For sample 4 (Figure 6), both the NOVA View and the operator classified this as a centromere pattern.



(a) (b)

Figure 6. Sample 4 HEp-2 Indirect Immunofluorescence (IIF) images, wide-angle view showing both interphase and metaphase cells, (b) enlarged metaphase cell (as indicated by the red arrow).

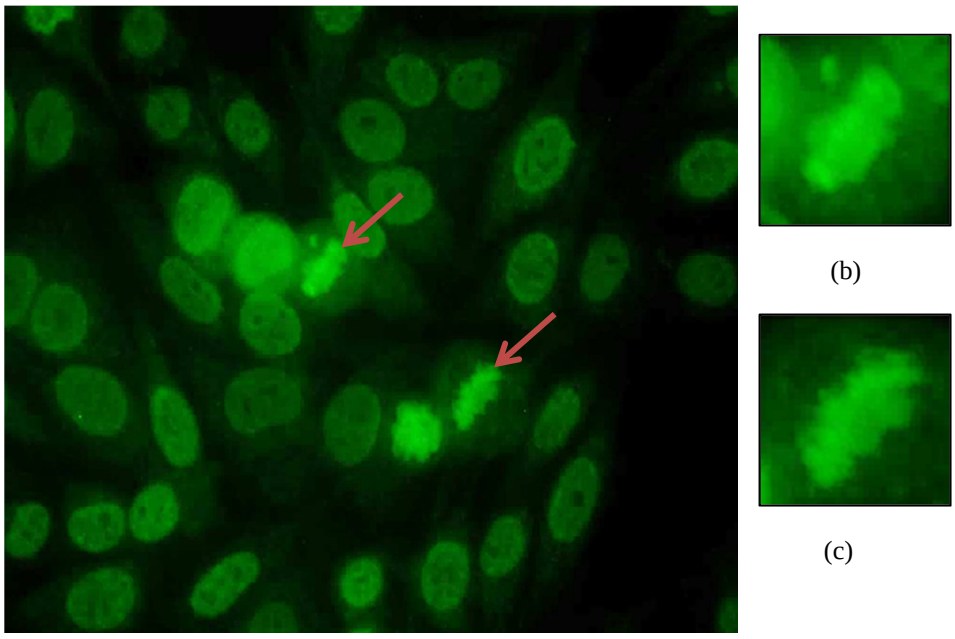
For sample 5 (Figure 7), while there does appear to be faint IIF staining, the LIU was below the cut-off for positivity and was therefore classified as ANA negative.



(a) (b) (c)

Figure 7. Sample 5 HEp-2 Indirect Immunofluorescence (IIF) images, (a) wide-angle view showing both interphase and metaphase cells, (b) and (c) enlarged metaphase cells (as indicated by the red arrows).

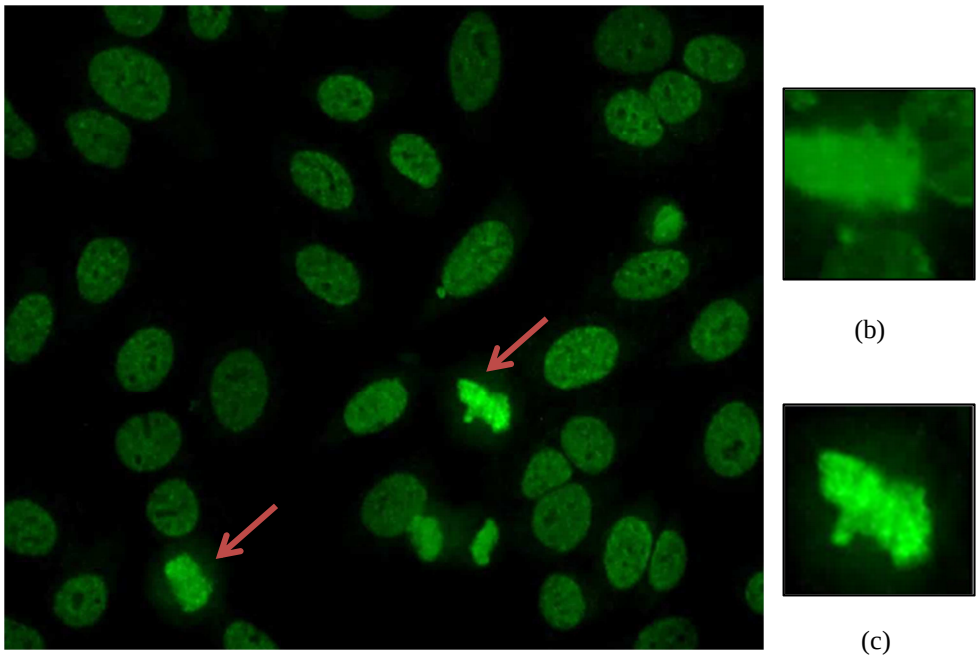
For sample 6 (Figure 8), both the NOVA View and the operator classified this as a homogenous pattern.



(a) (b) (c)

Figure 8. Sample 6 HEp-2 Indirect Immunofluorescence (IIF) images, (a) wide-angle view showing both interphase and metaphase cells, (b) and (c) enlarged metaphase cells (as indicated by the red arrows).

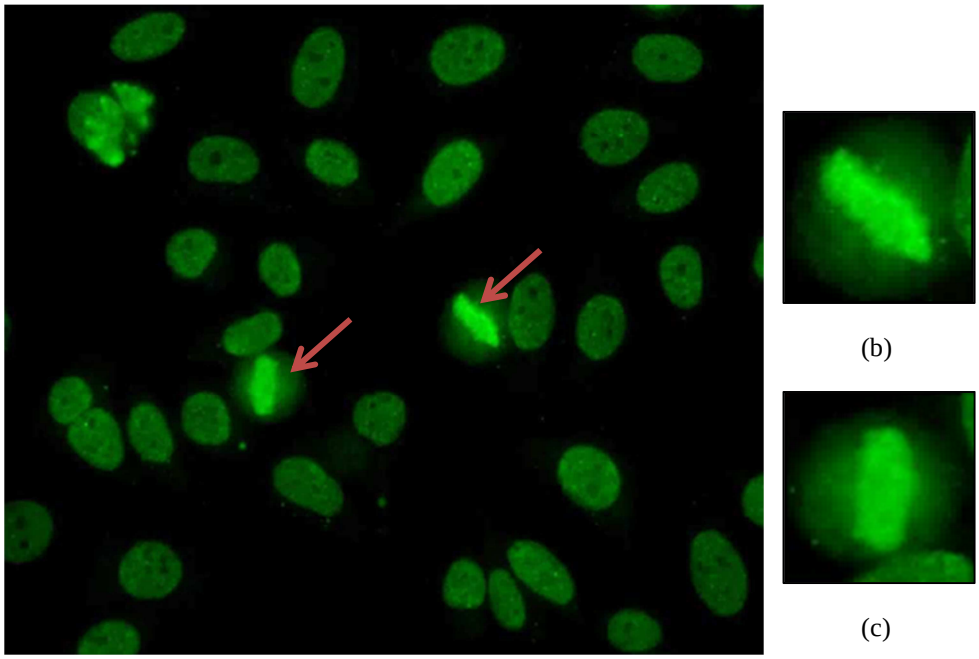
For sample 7 (Figure 9), the NOVA View was unable to classify this pattern, probably due to the speckled appearance with a positive staining mitotic cell as speckled patterns should have negative staining mitotic cells. The operator classified this as the dense fine speckled (DFS) pattern.



(a) (b) (c)

Figure 9. Sample 7 HEp-2 Indirect Immunofluorescence (IIF) images, a) wide-angle view showing both interphase and metaphase cells, (b) and (c) enlarged metaphase cells (as indicated by the red arrows).

For sample 8 (Figure 10), both the NOVA View and the operator classified this as a homogenous pattern.



(a) (b) (c)

Figure 10. Sample 8 HEp-2 Indirect Immunofluorescence (IIF) images, a) wide-angle view showing both interphase and metaphase cells, (b) and (c) enlarged metaphase cells (as indicated by the red arrows).

Thus, the presence of anti-DFS70 antibodies was not exclusive to the DFS pattern as anti-DFS70 antibodies were also found in samples exhibiting homogenous, speckled and centromere patterns. Table 4 shows which percentages of these were also positive for anti-DFS70 in the study cohort that was ANA positive by IIF.

Table 4.

Anti-DFS70 antibody distribution by the main ANA HEp-2 indirect immunofluorescence (IIF) patterns in the study cohort.

Anti-DFS70 presence	Homogenous pattern	Speckled pattern	Centromere pattern
DFS70 -	78	87	9
DFS70 + (%)	4 (4.8%)	1 (1.1%)	2 (18.1%)
Total	82	88	11

Note. DFS = dense fine speckled

The highest percentage was seen in the centromere pattern. Anti-DFS70 was also present in a sample showing a mixed homogenous / centromere IIF pattern (Sample 1). Sample 7 was the only sample to exhibit the classic DFS pattern as can be seen in Figure 8. This was correctly recognised by the operator, whereas the NOVA View classified it as ‘unrecognised’. The two anti-DFS70 positive samples that showed a centromere pattern (Sample 1 and Sample 4) had the presence of anti-centromere antibodies confirmed by ELISA (Table 3).

3.6 Prevalence of anti-DFS70 According to Age, Sex and Ethnicity

Table 5 shows that the majority of the cohort consisted of females in both the SARD and non-SARD groups and that of the eight anti-DFS70 positive samples, seven were from women and one from men. The highest prevalence of anti-DFS70 was seen in non-SARD females (7.8%) and the lowest prevalence was seen in non-SARD males (3.1%), however no significant difference between the sexes was observed (p = 0.5858).

Table 5.

Gender distributions of the SARD and non-SARD groups showing the prevalence of anti-DFS70 in each group.

Sex	DFS70 –	DFS70 + (%)	Total
Female	156	7 (4.3%)	163
Non-SARD	71	6 (7.8%)	77
SARD	85	1 (1.2%)	86
Male	47	1 (2.1%)	48
Non-SARD	31	1 (3.1%)	32
SARD	16	0 (0%)	16
Total	203	8	211

Note. DFS = dense fine speckled; SARD = systemic autoimmune rheumatic disease.

Table 6 shows the study cohort consisted mainly of patients older than 40 years old. The mean age of the anti-DFS70 positive patients was 44 years (range 16−82). The highest prevalence of anti-DFS70 was seen in the non-SARD patients of 10-40 years old age group (13%) and the lowest prevalence was seen in the non-SARD patients of the 60-80 years age group. However no association between the prevalence of anti-DFS70 and the age of individuals was observed (p = 0.4415).

Table 6.

Age ranges of the SARD and non-SARD groups with the prevalence of anti-DFS70 shown for each group.

Age range	DFS70 -	DFS70 + (%)	Total
10-40 Years	56	3 (5.1%)	59
Non-SARD	20	3 (13%)	23
SARD	36	0	36
10-40 Years	61	3 (4.7%)	64
Non-SARD	21	2 (8.7%)	23
SARD	40	1 (2.4%)	41
10-40 Years	71	1 (1.4%)	72
Non-SARD	46	1 (2.1%)	47
SARD	25	0	25
10-40 Years	15	1 (6.3%)	16
Non-SARD	15	1 (6.3%)	16
SARD	0	0	0
Total	203	8	211

Note. DFS = dense fine speckled; SARD = systemic autoimmune rheumatic disease.

Table 7 shows that the study cohort was represented by a number of ethnic groups, with the 4 main ethnic groups being NZ European (49.2%), Asian (19.4%), Other European (16.1%) and Pacific Islander (10.4%). Maori representation was 2.8% of the total cohort. All 8 anti-DFS70 positive samples were from New Zealand Europeans which was statistically significant (p = 0.0028). The highest prevalence of anti-DFS70 was seen in the non-SARD NZ European patients (13.5%).

Table 7.

Ethnic representation of the SARD and non-SARD groups with the prevalence of anti-DFS70 shown for each group.

Age range	DFS70 -	DFS70 + (%)	Total
Asian	41	0	41
Non-SARD	23	0	23
SARD	18	0	18
Middle Eastern	2	0	2
Non-SARD	2	0	2
SARD	0	0	0
Maori	6	0	6
Non-SARD	1	0	1
SARD	5	0	5
Pacific Islander	22	0	22
Non-SARD	8	0	8
SARD	14	0	14
NZ European	96	8 (7.7%)	104
Non-SARD	45	7 (13.5%)	52
SARD	51	1 (1.9%)	52
Other European	34	0	34
Non-SARD	22	0	22
SARD	12	0	12
Other	2	0	2
Non-SARD	1	0	1
SARD	1	0	1
Total	203	8	211

Note. DFS = dense fine speckled; SARD = systemic autoimmune rheumatic disease; NZ = New Zealand.

Table 8 summarises the anti-DFS70 positive patient demographics according to age, sex and ethnicity.

Table 8.

Demographics of the anti-DFS70 positive individuals.

Sample	Age	Sex	Ethnicity
1	82	F	NZ European
2	17	F	NZ European
3	16	M	NZ European
4	69	F	NZ European
5	48	F	NZ European
6	25	F	NZ European
7	57	F	NZ European
8	41	F	NZ European

Note. NZ = New Zealand; F = female; M = male

3.7 RCPAQAP Results

The two known anti-DFS70 positive RCPAQAP samples were both positive by ANA ELISA and IIF (Table 9). The NOVA View classified both as 'unrecognised', whereas the operator classified one as homogenous and the other as DFS. The anti-DFS70 CIA assay detected the anti-DFS70 in both RCPAQAP samples.

Table 9.

Royal College of Pathologists of Australia Quality Assurance Programme (RCPAQAP) results.

Sample	ANA IIF pattern NOVA View interpretation	ANA IIF pattern operator interpretation	ANA ELISA / ANA#	Anti-DFS70 CIA (CU)
RCPAQAP 1	Unrecognised	Homogenous	Positive 1.44	142
RCPAQAP 2	Unrecognised	DFS	Positive 1.27	127

Note. RCPAQAP = Royal College of Pathologists of Australia Quality Assurance Programme; ANA = antinuclear antibody; IIF = indirect immunofluorescence; DFS = dense fine speckled; CIA = chemiluminescence immunoassay; CU = chemiluminescent units.

3.8 Differences in the Referring Pattern of ANA Samples

As the study was performed in a general hospital, ANA requests were sent from a variety of clinical specialties and not from rheumatology alone (Table 10). Of the eight anti-DFS70 positive patients, the SARD patient was the only request received from rheumatology. Three of the non-SARD requests were received from Gastroenterology.

Table 10.

Referring departments of the anti-DFS70 positive patients.

Sample	Referring specialty
1	General Medicine
2	Dermatology
3	Gastroenterology
4	Otorhinolaryngology
5	Gastroenterology
6	General Medicine
7	Gastroenterology
8	Rheumatology

4 Discussion

4.1 Detection of anti-DFS70

4.1.1 ANA IIF vs ANA ELISA

Objective 1 of this study was to determine if the current ANA testing methods are adequately detecting anti-DFS70 antibodies, therefore all ANA testing was performed using both an IIF and ELISA technique. As discussed previously, both of these assays have some limitations therefore ideally all the samples in this study should have been tested using a number of ANA ELISA and IIF kits from different manufacturers, however this was not possible due to time and budget constraints. Nevertheless, the results showed that the Bio-Rad ANA ELISA kit is capable of detecting anti-DFS70 antibodies as all eight of the anti-DFS70 positive samples were ANA positive by ELISA. The ANA NOVA Lite® HEp-2 ANA kit with DAPI produced a negative ANA result for one of these samples (Sample 5). The corresponding ELISA result for Sample 5 was only just above the cut-off for positivity and so it may be that repeating the ELISA test could easily have produced a negative result as well. Sample 7 was also just above the cut-off for positivity for ELISA and so a repeat testing of this sample could also have yielded a negative result. Therefore it appears that the ELISA and IIF ANA screening assays are equally capable of detecting anti-DFS70 antibodies. Interestingly, anti-DFS70 antibodies have previously been detected in ANA negative samples, as reported by Bizzaro et al. (2015), although they did not state which ANA HEp-2 IIF kit they used. They tested for anti-DFS70 on a consecutive series of 155 patients sent for routine ANA testing by the CIA QUANTA Flash® DFS70 method, the same CIA method used in this study. Just two of the 155 patient samples were positive for anti-DFS70 and both of these were ANA negative. This suggests that the prevalence of anti-DFS70 in the general population is actually higher than that reported as anti-DFS70 is usually tested on ANA positive samples only.

Of interest, even though the ANA HEp-2 IIF assay supposedly has superior sensitivity to the ELISA ANA screening assay, in this study there were considerably more ELISA positive / IIF negative results (24) than there were ELISA negative / IIF positive results (8). In addition, three of the ELISA positive / IIF negative results had a positive ENA, whilst none of the ELISA negative / IIF positive results had a positive ENA. There is

evidence that some of the newer ANA screening technologies assays have superior sensitivity to the ANA HEp-2 assay (de Almeida Brito, et al. 2016), however further comparisons are required in order to determine if this is the case here.

4.1.2 NOVA View

An automated IIF slide reader was used in an attempt to standardise the IIF results and therefore reduce subjective interpretation. While it is known that the NOVA View cannot specifically identify the DFS pattern, the purpose of this study was to determine if the NOVA View was capable of flagging the DFS pattern as ANA positive in order to alert the operator that a suspected rare pattern is present. The only sample to produce the classic DFS pattern was Sample 7 which the NOVA View flagged as 'unrecognised'. The two RCPAQAP samples were also classified as such by the NOVA View therefore it appears that it is capable of detecting the DFS pattern. It is important to note that the NOVA View did not incorrectly classify the DFS pattern as either 'speckled' or 'homogenous'. By calling the DFS pattern 'unrecognised', it alerted the operator to closely inspect the pattern as opposed to quickly confirming the NOVA View result. Although in saying this, this was not the case in an experiment by Bizzaro et al. (2013) where six different automated slide readers, including the NOVA View, incorrectly classified the DFS pattern as either speckled or homogenous (Bizzaro et al., 2015). During visual validation by the operator, a useful feature of the NOVA View is that it shows 2-3 enlarged IIF images of the mitotic cells. This is important for DFS pattern classification as the type of staining on the mitotic cells is essential for DFS pattern recognition and helps to differentiate this pattern from a traditional speckled pattern. This feature of the NOVA View may therefore help the operator to better identify the DFS pattern than by standard IIF microscopy.

With the need to increase the throughput of the IIF assay and standardise pattern interpretations there will almost certainly be an increased use of automated IIF slide readers in clinical laboratories. With additional features such as cumulative review of images, remote reporting and quality control features, the use of automated IIF slide readers has become even more appealing. Nevertheless, the results of this study as well as the Bizzaro et al. (2013) study highlight the importance of visual validation of pattern interpretations made by automated slide readers. In particular all 'unrecognised' patterns should be closely inspected by the operator and the operator should keep in mind that a DFS pattern may be present.

4.1.3 Anti-DFS70 and the DFS IIF pattern

The identification of the DFS pattern was found to be quite challenging and the RCPAQAP results show that this appears to be the case for most of the laboratories participating in the programme. For RCPAQAP1, of the 90 laboratories that submitted a result, just 25 correctly identified the DFS pattern. 50/90 laboratories classified it as a speckled pattern and 50/90 classified it as homogenous either in isolation or with another pattern (RCPAQAP Immunology, 2015a). For RCPAQAP2 just 25/94 laboratories correctly identified the DFS pattern, with 64/94 classifying it as a homogenous pattern and 24/94 classifying it as a homogenous / speckled pattern (RCPAQAP Immunology, 2015b). In the cases where other patterns or auto-antibodies were present, the DFS pattern may actually have been masked. Sample 1 appears to show a mixed centromere / homogenous pattern however, this could easily be confused with a mixed centromere / DFS pattern. This was shown to be the case in the Bentow et al. (2016a) study where a picture of a mixed serum 50:50 DFS / centromere pattern was presented for interpretation. Just 10% of respondents were able to correctly identify the mixed pattern, whereas 27% of respondents thought it was a homogenous / centromere mixed pattern.

Anti-DFS70 was not exclusively present in samples exhibiting a DFS pattern only, but was found in all three of the main IIF patterns. Table 2 shows that the highest incidence of anti-DFS70 was seen in the samples exhibiting a centromere pattern (18%) followed by the homogenous (5%) and then speckled (1%) patterns. Gundin et al. (2016) also reported the presence of anti-DFS70 antibodies in other IIF patterns; however they reported a higher incidence in homogenous (14.7%) and speckled (11.5%) patterns, with no anti-DFS70 antibodies detected in the centromere pattern. They offered no possible explanation for their findings. It may be that some of these patterns have been incorrectly classified by the technologist or perhaps the DFS pattern is present but is being masked by the other patterns. As most previous studies tested for anti-DFS70 on samples presenting with a DFS pattern only, this could mean that the prevalence of anti-DFS70 may be higher than that which has been reported.

In order to confirm the presence of the DFS pattern in possible mixed patterns, an anti-DFS70 inhibition assay could be used. Inhibition works by blocking the auto anti-DFS70 antibodies and thus preventing them from binding to their target antigen on the

HEp-2 substrate. By preventing the detection of the DFS pattern, any other patterns present would be clearer to interpret (Bentow et al., 2016b). For this study, had an inhibition assay been performed on the anti-DFS70 positive samples it would have clearly shown whether or not the DFS pattern was present in each case. For example, Sample 2 was strongly positive for anti-DFS70 (>450CU) yet the NOVA View gave an 'unrecognised' IIF pattern and the operator has interpreted it as a speckled pattern. Figure 4 shows that for Sample 2, the NOVA View has highlighted 2 mitotic cells with one showing a clear negative staining pattern, whereas the other appears to be showing a positive staining pattern which is probably why the NOVA View gave an 'unrecognised' result. Sample 2 also had anti-Scl70 present, which often presents as a speckled pattern by IIF (Aggarwal, 2014). Therefore it is quite possible that in this case there is in fact a mixed speckled / DFS pattern and so an inhibition assay would have confirmed this. Any future studies should include anti-DFS70 inhibition assays in order to confirm the presence of the DFS pattern and to identify any underlying mixed patterns. Alternatively, if one can conclude that anti-DFS70 antibodies are found in samples exhibiting other IIF patterns, then testing all ANA positive samples for anti-DFS70 antibodies regardless of the IIF pattern presented would be the preferred option, which was the protocol for this study. Since this has not been the case in most previous studies, this could mean that the prevalence of anti-DFS70 may be higher than that which has previously been reported.

4.1.4 Anti-DFS70 CIA assay

As previously stated, anti-DFS70 was detected by using the QUANTA Flash® DFS70 CIA (Inova Diagnostics) as most previous studies had used this assay therefore differences between prevalences could be better compared. However it is possible that this assay does not detect all anti-DFS70 antibodies. It could be that some anti-DFS70 antibodies recognise different targets in the DFS70 antigen and since this assay uses a recombinant DFS70 antigen (INOVA Diagnostics, 2013), there may be missing epitopes that some of these anti-DFS70 antibodies are directed against. Although if this is the case then it would be a small minority of anti-DFS70 antibodies not being detected. Mahler et al. (2012) compared the QUANTA Flash DFS70® CIA with an anti-DFS70 ELISA assay and they found that 52/53 samples were positive for anti-DFS70 antibodies by CIA, whereas the ELISA assay was positive for all 53 samples. They did however show an excellent quantitative correlation between the two methods. Even

though it would only be a minority of anti-DFS70 antibodies not being detected by CIA, ideally two different methods for anti-DFS70 detection should have been used in this study as this would have ensured that all anti-DFS70 antibodies were correctly detected. However this was not possible due to the limited budget. In order to provide some assurance that anti-DFS70 was being detected, two known anti-DFS70 positive RCPAQAP samples were tested and both were positive for anti-DFS70. This therefore added some external quality control to the study.

4.2 Prevalence of anti-DFS70

Objective 2 of this study was to determine the local prevalence of anti-DFS70 in ANA positive patients in a New Zealand public hospital population. Due to time and budget constraints, this study was limited to testing 100 each of SARD and non-SARD patients so that the prevalence of anti-DFS70 in each group could be compared. In order to determine the overall prevalence of anti-DFS70 in ANA positive patients, a number of consecutive ANA positive samples would have had to be tested regardless of their SARD status. As this study took place in a public hospital setting, ANA requests were received not only from Rheumatology but from other specialties and general wards as well. Rheumatology clinics are run at certain times of the month therefore the ratio of SARD to non-SARD patients being tested for ANA does fluctuate in this laboratory. This means that testing for the prevalence of anti-DFS70 in all ANA positive patients may have given different results depending on when the samples were collected. Any large future studies would need to test for anti-DFS70 on all ANA positive samples for a period of a few months in order to determine the prevalence of anti-DFS70 amongst all ANA positive patients.

4.2.1 Prevalence of anti-DFS70 in SARD and non-SARD groups

The local prevalence of anti-DFS70 in ANA positive non-SARD patients was significantly higher than those with known SARD. This finding was expected and is consistent with previously published reports (Dellavance et al., 2005). The prevalence of anti-DFS70 in SARD patients was just 1%, which indicates that anti-DFS70 antibodies are rarely found in patients with SARD and provides further evidence that the presence of anti-DFS70 makes a SARD diagnosis unlikely. It is not certain as to why anti-DFS70 is more prevalent in non-SARD patients. A possible cause is the type of treatments SARD patients are on (Miyara et al., 2013). As SARD patients are likely

to be on immune suppressing drugs, it is possible that these drugs are preventing anti-DFS70 production. This in turn would mean that newly diagnosed SARD patients not on any treatments would be more likely to have anti-DFS70 antibodies present. This study did not investigate the types of treatment the SARD and non-SARD patients were on at the time of sample collection. A larger, more in-depth study is required in order to determine if there is an association between certain drugs and anti-DFS70 positivity.

The prevalence of anti-DFS70 in SARD patients obtained in this study is less than expected as the literature has reported prevalences upwards of 5% (Muro et al., 2008, Bizzaro et al., 2015). The lower prevalence obtained here may due to a number of reasons. This study used two different ANA detection methods in order to increase the likelihood of anti-DFS70 detection, therefore it is unlikely that the cause is due to the inherent technical limitations of either assay resulting in false negative ANA results and thus a reduced detection of anti-DFS70. This study has confirmed that of previous studies in that the DFS pattern is challenging to identify and that anti-DFS70 antibodies can also be found in samples exhibiting other IIF patterns. Since all the ANA positive samples were tested for anti-DFS70 in this study, these factors have been eliminated as possible causes of the difference in prevalence obtained here. It may be that the DFS70 epitope used in the detection assay is not detecting the anti-DFS70 sufficiently. However the same anti-DFS70 CIA detection assay was used in the Bizzaro et al. (2015) study and they reported a prevalence of 7.5% in SARD patients, which suggests that this may also not be an influencing factor. It is likely that the difference in the prevalence of anti-DFS70 in SARD patients is due to differences in the cohorts used in each study. As a consecutive series of samples was used in this study, the cohort consisted of random patients. Therefore an imbalance in patient demographics could be the reason. Other possible influencing factors include inappropriate ANA testing from clinicians, patient treatments and environmental factors. Interestingly, Bizzaro et al. (2015) summarised the prevalence of anti-DFS70 obtained by various study groups in different disease states and in some cases the prevalences within each disease group vary significantly. For example, four studies have reported a prevalence of anti-DFS70 in atopic dermatitis patients and their results vary significantly (0%, 7%, 30% and 38%). It is possible that there may be multifactorial reasons as to why different prevalences have been obtained.

The prevalence of anti-DFS70 found in the non-SARD patients in this study could not be directly compared with the literature as no previous studies have reported this value directly. For example, Dellavance et al. (2005) reported that 39% of their DFS pattern positive patients were SARD and 61% were non-SARD and from this they concluded that the prevalence of anti-DFS70 in ANA positive non-SARD patients was significantly higher than those with known SARD. Bizzaro et al. (2015) used 155 consecutive samples sent by referring physicians for routine ANA testing, but they did not provide further information such as how many of these were ANA positive, and of the ANA positive patients they did not state how many were SARD and non-SARD. Therefore the prevalence of each group in a routine clinical setting could not be determined in their study. Instead they reported the prevalence of anti-DFS70 for the entire group, which was 1.3%.

4.2.2 Prevalence according to age, sex and ethnicity

A research question for Objective 2 was to determine if there is a significant difference in the prevalence of anti-DFS70 according to age, sex or ethnicity. This study aimed to determine the prevalence of anti-DFS70 in a New Zealand general hospital population. As previously stated, most of the published anti-DFS70 studies had been performed in countries where the study population had mostly been limited to a single ethnic group. However Auckland, New Zealand is unique in that due to the high levels of immigration in recent years, the population consists of a number of ethnic groups, the majority of which are European (59%), Asian (23%), Pacific Islander (15%) and Maori (10%) (Auckland Council, 2014). Even though more than half of this study's cohort consisted of NZ Europeans, other ethnic groups were represented in similar proportions to the above statistics (Table 6). This study did not allow for equal representation of all ethnic groups as the samples used were a consecutive series of patient samples. Since all eight samples positive for anti-DFS70 were from NZ European patients this indicates that the presence of anti-DFS70 may have a genetic or possibly a geographic component. Future studies with an increased sample size focusing on each ethnic group would help to clarify this.

No significant differences between age and sex in the prevalence of anti-DFS70 were observed, a finding which differs to that of the majority of previous studies as discussed previously. However, Mahler et al. (2012) also did not find a significant difference in the prevalence of anti-DFS70 according to age or sex and so this result is not an isolated

case. The gender distribution for this study included significantly more females than males (Table 4) however this was the case for all previous studies as SARD conditions are more likely to occur in females (Mahler et al., 2012) and hence more females are being tested for ANAs. Future studies could attempt to stratify the cohort by sex in order to confirm the association of anti-DFS70 antibodies and sex. Although the study cohort had a wide age range (13-98 years), the age distribution of the cohort (Table 5) consisted mostly of patients over 40 years of age. Future studies should attempt to include an equal age range distribution in the cohort in order to confirm whether or not the prevalence of anti-DFS70 antibodies changes according to age.

4.2.2.1 Maori health

The New Zealand Health Strategy has made Maori a priority group as their health outcomes are generally poorer than the non-Maori populations (Ministry of Health, 2016). Therefore it is important to note the health outcome for Maori, if any, in this study. It was difficult to estimate what the percentage of Maori representation would be for this study as there is no data on the prevalence of SARD in Maori available. However, since Maori represent 10% of the Auckland population (Auckland Council, 2014) and since in 2011/2012 16.4% of publicly funded hospital discharges were Maori (Ministry of Health, 2014), (although the study cohort did also include outpatient samples), it was estimated that the cohort would consist of about 10-15% Maori. Unfortunately this would not have been an adequate representation in order to draw any definitive conclusions on the prevalence of anti-DFS70 in Maori. As the study cohort numbers were limited and a consecutive series of patients were selected, it was out of the scope of this study to select samples according to ethnic groups in order to ensure adequate Maori representation. Any future studies should attempt to over represent Maori in their studies in order to address equity issues. This principle of 'equal explanatory power' has been suggested an acceptable practice as it allows data for Maori and non-Maori to be analysed equally so that any differences in health outcomes can be noted for each group (Te Rōpū Rangahau Hauora a Eru Pōmare, 2015).

Ultimately, just 3% of the cohort in this study consisted of Maori, a figure far below the estimated percentage. Whilst none of the Maori in the cohort had anti-DFS70, no conclusions or assumptions could be made on such a low number of samples.

4.3 Clinical Significance of anti-DFS70

Objective 3 was to determine if the presence of anti-DFS70 antibodies is clinically significant in terms of a SARD diagnosis. The prevalence of anti-DFS70 was significantly higher in non-SARD patients than in SARD patients, which tends to indicate that the presence of anti-DFS70 makes a SARD diagnosis unlikely. The non-SARD patients with anti-DFS70 had a variety of conditions with no obvious common symptoms (Table 1), therefore the presence of anti-DFS70 cannot be linked to any non-SARD condition either as it has been in the past. For example, anti-DFS70 antibodies have been linked to atopic dermatitis, interstitial cystitis and autoimmune thyroiditis (Dellavance et al., 2005), yet none of the eight anti-DFS70 positive patients in this study displayed symptoms of any of these conditions.

4.3.1 Anti-DFS70 and ENAs

The presence of other ENAs in conjunction with anti-DFS70 may also be a deciding factor as to whether or not the presence of anti-DFS70 can be used to possibly eliminate a SARD diagnosis. The majority of the non-SARD anti-DFS70 positive patient samples did not have any other ENAs present, however neither did the single SARD patient to test positive for anti-DFS70. This SARD patient had a history of negative ENAs with a positive IIF ANA displaying a homogenous pattern and had been diagnosed with SLE many years previously. While this is an interesting finding, Mahler et al. (2012) also reported a case where an SLE patient with anti-DFS70 antibodies did not have any other autoantibodies present. Because of this, it cannot be concluded that the presence of an isolated anti-DFS70 can be used to completely eliminate a SARD diagnosis. These results rather point to an unlikely SARD diagnosis.

Two of the eight anti-DFS70 positive samples also had anti-centromere antibodies present. Anti-centromere antibodies are usually found in 50-80% of patients with limited systemic sclerosis (Aggarwal, 2014). For Sample 1, this patient had a history of anti-centromere antibodies with a diagnosis of lung cancer and primary sarcoidosis so it appears that the presence of anti-centromere antibodies here is an aberrant finding.

Sample 4 had anti-centromere antibodies as well as anti-SSA present. Antibodies to SS-A/Ro have been detected in approximately 25–30% of patients with SLE and 40–70% of patients with Sjögren's Syndrome but has also been seen in other autoimmune

conditions (Aggarwal, 2014). With no previous ANA history, the clinicians concluded that this patient did not have a SARD condition, although whether this patient does develop SARD in the future is yet to be seen. Anti-SSA typically presents as a speckled pattern by IIF (Aggarwal, 2014) which does not appear to have manifested here.

Sample 2 also had anti-Scl70 present. Antibodies to Scl-70 are often present in patients with diffuse systemic sclerosis (Aggarwal, 2014). This patient had a history of anti-Scl70 antibodies with symptoms of recurrent blistering but had yet to be diagnosed with a SARD condition. A follow up is required in order to determine if the patient does develop one in the future. Anti-Scl70 usually presents as a speckled pattern by IIF (Aggarwal, 2014), which is consistent with the results obtained here.

Sample 6 was interesting in that it was strongly positive for dsDNA antibodies with the Autoimmune EIA anti-dsDNA Test (Bio-Rad) assay but it was negative by the Farr assay. The Farr assay detects high-avidity antibodies only whereas ELISA detects both low-affinity and high-affinity antibodies. However due to the lower specificity of the ELISA assay, the Farr assay is the method of choice recommended by an international panel. Nevertheless ELISA is the most widely used as it is easy to perform and it also has no radiation hazard (Aggarwal, 2014). Anti-dsDNA antibodies are present in two-thirds of patients with SLE, however due the negative Farr result and the lack of clinical symptoms, the clinicians concluded that this patient did not have SLE.

Thus it appears that the presence of other ENAs in conjunction with anti-DFS70 are aberrant findings and do not indicate a SARD condition. A larger sample size and follow up of these patients in order to determine whether or not they eventually develop a SARD condition is required in order to confirm this. However, the results of this study appear to indicate that anti-DFS70 with or without the presence of other ENAs make a SARD diagnosis highly unlikely. These cases also highlight the fact that the patient's clinical signs and symptoms should always be considered in SARD diagnosis, regardless of the autoantibody results.

4.3.2 Clinical significance of anti-DFS70 CIA positivity

A research question for Objective 3 was 'Is the amount of anti-DFS70 detected clinically significant in terms of a SARD diagnosis?' This question does not appear to be addressed in any of the literature to date but was worth investigating as it may be that an anti-DFS70 CU value above or below a certain threshold would make a SARD

diagnosis more or less likely. The QUANTA Flash® DFS70 assay is semi-quantitative and the amount of CU produced is proportional to the amount of antibody in the patient sample (INOVA Diagnostics, 2013). The amount of anti-DFS70 in the positive patient samples ranged from 33CU to greater than 450CU (mean=193CU), with the SARD patient having a value of 137CU. Therefore there does not appear to be any obvious correlation between the amount of anti-DFS70 present and a SARD diagnosis, however further studies with a larger sample size would be required to confirm this.

4.3.3 Incorporating anti-DFS70 into the ANA test algorithm

If we can conclude that the presence of anti-DFS70 in ANA positive patients makes a SARD diagnosis highly unlikely, particularly when no ENAs are also present, then the anti-DFS70 assay should be incorporated into the ANA test algorithm and the result should be reported to the clinicians. Currently, most laboratories perform the HEp-2 ANA test for the initial ANA screening and will then go on to perform an ENA panel (including dsDNA) as shown in Figure 11. The IIF pattern, titer and type of ENA present would then provide the clinician with a likely SARD diagnosis.

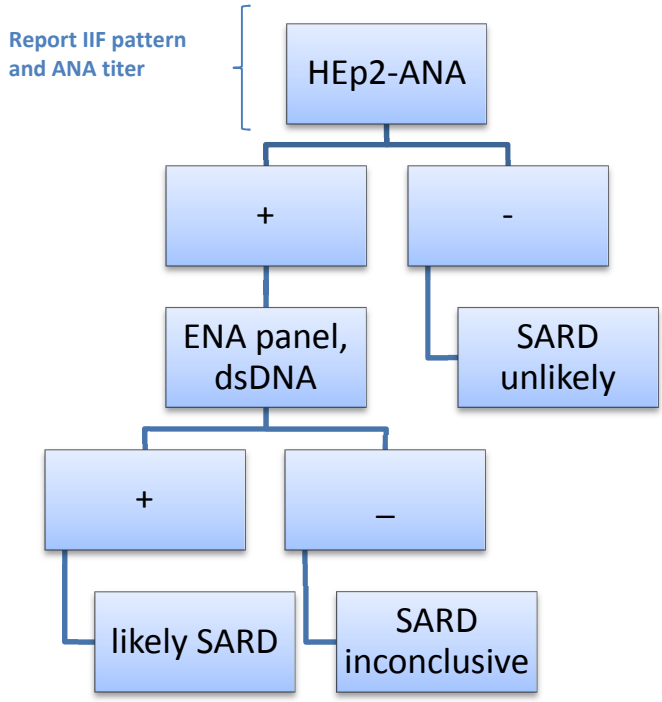


Figure 11. Conventional antinuclear antibody (ANA) test algorithm. The immunofluorescence (IIF) pattern, antibody titre and type of extractable nuclear antigen (ENA) present will usually provide a likely systemic autoimmune rheumatic disease (SARD) diagnosis.

If anti-DFS70 was also tested for, then the ANA result interpretation would likely be that as proposed in Figure 12.

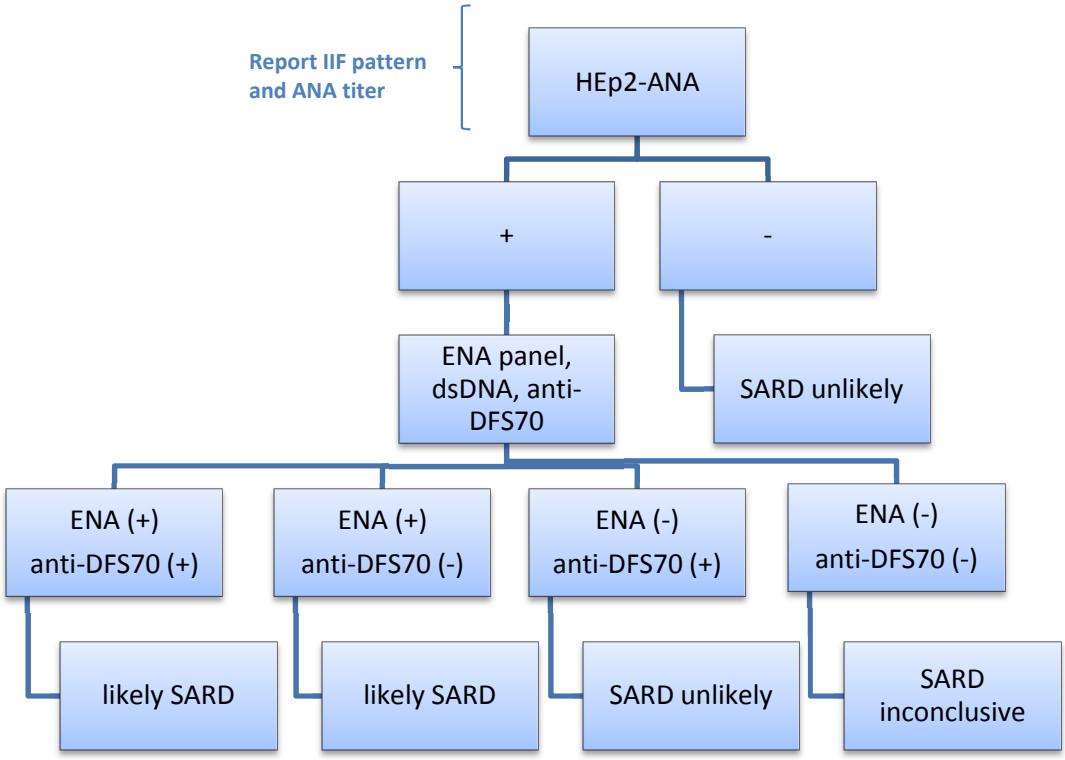


Figure 12. Proposed new antinuclear antibody (ANA) result interpretation algorithm that includes the anti-dense fine speckled (DFS)70 result. Patients with a positive extractable nuclear antigen (ENA) in combination with either a positive or a negative anti-DFS70 result have an increase likelihood of having systemic autoimmune rheumatic disease (SARD). Patients with a negative ENA and a positive anti-DFS70 result have a low likelihood for having SARD. Patients with a negative ENA and a negative anti-DFS70 result are inconclusive for a SARD diagnosis. These patients should have follow up testing.

In this new algorithm, results that are ENA positive would continue to be a likely SARD diagnosis, regardless of the anti-DFS70 result. Results that are ENA negative, anti-DFS70 negative would indicate that a SARD diagnosis is highly unlikely and results that are ENA negative, anti-DFS70 positive would indicate that a SARD diagnosis is inconclusive. These patients would require monitoring and follow up testing in order to determine if their ENA status changes.

When to test for anti-DFS70 in the ANA test algorithm has not yet been agreed upon in the literature, however there is the cost factor to consider when making this decision. Costs are important to consider as spiralling healthcare costs have become an increasing problem worldwide, including in New Zealand (Ministry of Health, 2012). Most studies have tested for anti-DFS70 only on those samples exhibiting a DFS pattern in order to merely confirm the presence of anti-DFS70. While this may keep costs down, this

present study has shown that the DFS pattern is difficult to clearly identify and that anti-DFS70 can be found in other IIF patterns as well. Therefore ideally anti-DFS70 should be tested on all ANA positive samples. Then again, if the anti-DFS70 result adds no value to the diagnostic interpretation of ENA positive samples, then there is no need to test for it in patients with a positive ENA. By testing for anti-DFS70 in just those ANA positive samples that are ENA negative, costs would be kept to a minimum and in terms of a SARD diagnosis, the anti-DFS70 result would provide the greatest value here (Figure 13).

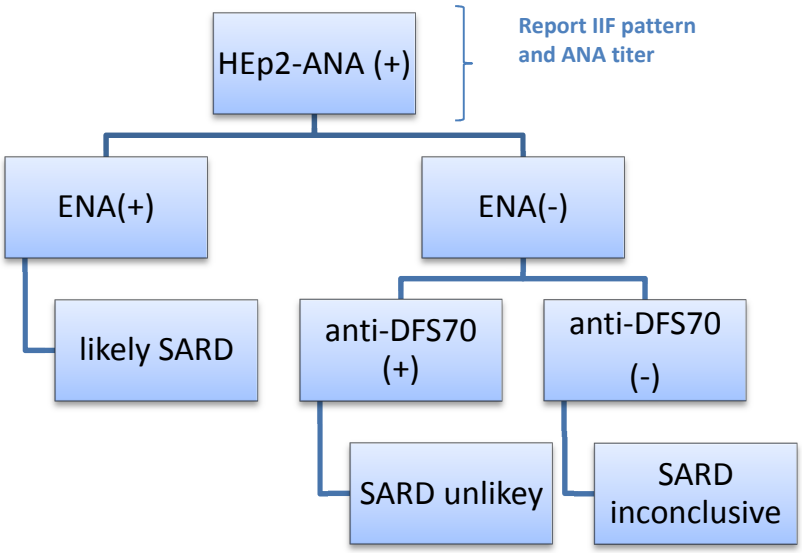


Figure 13. Proposed new antinuclear antibody (ANA) test algorithm considering anti-dense fine speckled (DFS)70 antibodies. Anti-DFS70 should be tested for on all ANA positive, extractable nuclear antigen (ENA) negative samples regardless of the immunofluorescent (IIF) pattern seen. Patients with a negative ENA and a positive anti-DFS70 result have a low likelihood for having systemic autoimmune rheumatic disease (SARD). Patients with a negative ENA and a negative anti-DFS70 result are inconclusive for a SARD diagnosis. These patients should have follow up testing.

Ultimately, where in the ANA test algorithm anti-DFS70 is to be tested for would depend on each individual diagnostic laboratory, as each laboratory would need to consider the work flow, costs and turnaround times of their entire ANA testing process. Some laboratories may decide to reduce costs and wait for the ENA result before testing for anti-DFS70 as in Figure 13. Others may not want to sacrifice turnaround times, or they may wish perform the anti-DFS70 test regardless of the ENA result so that the clinicians can decide on the significance of the anti-DFS70 result. These laboratories would thus test all ANA positive samples for anti-DFS70, as in Figure 12.

4.4 Appropriateness of the ANA Test

Multiple studies have shown that there is a high ANA positivity rate within the general population. At a dilution of 1:80, the ANA positivity rate can be as high as 50% and even at a dilution of 1:160 the positivity rate has been shown to still be fairly high at around 9.5% (Abeles & Abeles, 2013). Thus with the ANA HEp-2 assay having such a limited specificity, the ANA test can be problematic when it is not appropriately requested and becomes even more troublesome when there is an incomplete understanding of how to interpret the findings.

As has been briefly discussed previously, ANA test referral patterns have changed in recent years. With requests coming from other specialties and not only from Rheumatology, there has been a significant increase in the number of ANA positive patients that do not have SARD (Mahler & Fritzler, 2012). This was highlighted in a recent study where more than 90% of the patients with a positive ANA result that were referred to a tertiary Rheumatology clinic had no clinical evidence of an ANA-associated SARD (Abeles & Abeles, 2013) . The consequences of this are that a large number of patients are having unnecessary follow up investigations and treatments and hence it is important that clinicians request the ANA test appropriately.

If the presence of anti-DFS70 can infer that a SARD diagnosis is highly unlikely, then this would not only reduce the amount of anxiety suffered by the patients, but also the costs involved in the follow up of these patients. Table 8 shows that only the single SARD patient with a positive anti-DFS70 result had the ANA test requested by Rheumatology. The other requests came from a variety of specialties, which is in keeping with previous findings (Mahler & Fritzler, 2012). Interestingly, three requests came from Gastroenterology. This finding differs from a previous report by Miyara et al. (2013), where they found most anti-DFS70 positive samples came from Neurology and Hepatology.

These results highlight the fact that the ANA test should be appropriately tested for in the correct clinical setting. In retrospect, it would have been useful to have made a note of where all the ANA requests for this study had originated from in order to further highlight this point.

4.5 Strengths and Limitations

The results of this study should to be considered with caution as there were a few shortcomings, some of which have already been referred to above. As this is a University Masters thesis research paper, time and resources were limited and consequently sample size was restricted to 100 each of SARD and non-SARD patients. Thus for some of the research questions, the sample size was large enough to draw tentative conclusions from but in order to draw definitive conclusions, larger multi-centre studies are required. For instance, this is what is required in order to accurately determine whether or not the prevalence of anti-DFS70 changes according to age, sex and ethnicity, with specific attention to the Maori population required. On the other hand, since the 100 each of SARD and non-SARD patients was sequential samples of each, and since all the assays used were routine assays used in the laboratory, this reflects real-world practice which is actually a great strength of this study. Thus the sample size was sufficient to determine a 'snapshot' of whether or not anti-DFS70 is being detected in New Zealand laboratories, how often it is present in a New Zealand ANA positive hospital population and if its presence is interfering with the diagnosis of SARD.

Another shortcoming of this study is that there was no long-term follow up data on the study patients, thus it cannot be known for certain as to whether or not any of the ENA positive, anti-DFS70 positive patients would develop a SARD over time, especially since some SARD conditions do develop years after an ENA is first detected. For example, in some case anti-dsDNA and anti-Scl70 may appear in the blood for years before any symptoms of a SARD condition begin to manifest (Smeenk, 2000). In particular, for Sample 4, it would be interesting to see whether or not this patient develops a SARD in years to come as this person has both SSA and centromere antibodies present.

Only one commercial ANA IIF kit and ANA ELISA kit was used in this study. Since it is known that there are significant differences in the detection of ANAs between kits from different manufacturers, if a number of kits from different manufacturers were used in this study, this would have further increased the likelihood of ANA, and consequently anti-DFS70, detection. The use of two different methods for anti-DFS70 detection would also have ensured that all anti-DFS70 antibodies were detected. It

would also have been useful to have performed an inhibition assay on the anti-DFS70 positive samples in order to see if the DFS pattern was present.

On the other hand, this study also has some other noteworthy strengths. These include the fact that ANAs were detected using two different ANA detection methods and that anti-DFS70 was tested on all ANA positive patients and not just those exhibiting a DFS IIF pattern. Therefore the likelihood of detecting anti-DFS70 antibodies was greatly increased. The use of the NOVA View to read the IIF patterns helped to reduce some subjective interpretation as to whether a result was positive or negative and it also helped to interpret the main IIF patterns, whilst flagging any mixed or rare patterns. Thus the use of the NOVA View added some standardisation to the ANA IIF assay in this study which some previous studies have lacked.

5 Conclusions

Since the presence of anti-DFS70 antibodies has the potential to eliminate a SARD diagnosis in ANA positive patients, an objective of this study was to determine if this could be applied to the New Zealand population. In addition, I wished to determine if anti-DFS70 was currently being detected in routine ANA assays and what the prevalence of anti-DFS70 is in a New Zealand population. Thus I hoped to conclude whether or not New Zealand laboratories should be specifically testing for the presence of anti-DFS70 antibodies in ANA positive patients and reporting the result to the clinicians.

5.1 ANA Testing

During the course of this study, some of the limitations of the ANA screening test and the appropriateness of the request were highlighted. Even with its well-known shortcomings, the ANA IIF assay continues to be the Gold Standard assay for ANA detection. Whilst the ANA IIF assay does have a low specificity, newly developed ANA screening technologies have not yet achieved the same sensitivity as it. However, with the increased demand for diagnostic testing and rising healthcare costs worldwide, it seems unlikely that such a labour intensive assay with a low specificity will continue to be employed in years to come. Automated IIF slide readers have been developed in an attempt increase the throughput of the IIF assay and standardise pattern recognition, however this study has highlighted the fact that visual validation of results is still required as automated slide readers cannot recognise all patterns (such as the DFS pattern) and mixed patterns are also difficult to interpret.

This study has shown that ANA requests are being received from a variety of clinical specialties; therefore it is important that all clinicians and not just those with a rheumatology background are aware of the limitations of the ANA screening assay. Since results can vary between laboratories, the clinician should know the method used as well as the sensitivity and specificity of the assay in order to properly interpret the results. In addition, due to the reduced specificity of the ANA IIF assay, it is advisable that clinicians consider the appropriateness of the ANA test in the first instance as false positive ANA results can lead to clinician confusion and undesirable consequences for patients.

5.2 Anti-DFS70 Detection

This study has shown that anti-DFS70 antibodies are being detected by routine ANA assays in New Zealand diagnostic laboratories and that the NOVA View is capable of flagging the DFS pattern as positive. However, as pointed out in this study, recognising the DFS ANA IIF pattern and mixed IIF patterns poses a significant challenge and anti-DFS70 antibodies can be found in other IIF patterns as well. Therefore the detection of even an isolated anti-DFS70 by solely using IIF is likely not performed with high precision in New Zealand diagnostic laboratories. Even though the laboratories participating in the RCPAQAP survey are not exclusively New Zealand laboratories, the results of the RCPAQAP survey still highlight this point. Consequently, it seems imperative that all ANA positive samples should be tested for anti-DFS70 using an assay specifically designed to detect it, regardless of the IIF pattern it presents with. Alternatively, in order to confirm the presence of the DFS pattern, especially in possible mixed patterns, an anti-DFS70 inhibition assay could be employed. In addition, the new ANA IIF automated slide readers could be further improved if they were better able to recognize mixed patterns or less common patterns such as the DFS pattern.

5.3 Prevalence and Clinical Significance of anti-DFS70

The results of this study have confirmed that the prevalence of anti-DFS70 in a New Zealand population is significantly higher in non-SARD patients than in SARD patients. Also, anti-DFS70 antibodies are usually found in isolation in non-SARD patients. Thus, although the presence of anti-DFS70 antibodies cannot exclude a SARD diagnosis, the likelihood is significantly lowered, particularly if no other ENAs are present. While this study does not have any follow-up data, as mentioned previously there is evidence that patients with an isolated anti-DFS70 do not develop SARD in years to come. This thus eliminates the need for follow-up testing or unnecessary treatments for these patients. Therefore in conclusion, the anti-DFS70 assay should be included in the ANA test algorithm and the result should be included in the laboratory report. Where in the ANA test algorithm anti-DFS70 should be tested for should be up to the laboratory, as each laboratory would need to consider work flow, costs and turnaround times of their entire ANA testing process. Laboratory reports should include an appropriate interpretative comment clearly explaining the significance of the anti-DFS70 result as it is imperative that clinicians are aware of its significance in terms of SARD diagnosis. Clinicians

should be aware that where a patient has anti-DFS70 antibodies, they should rather focus on the presence or absence of other ENAs and more importantly, on the presence or absence of clinical signs and symptoms of SARD.

It was shown that the prevalence of anti-DFS70 does vary according to ethnicity, with no correlation found between age and sex. However this was likely due to the small sample size therefore further studies with a larger cohort would be required in order to confirm these findings.

5.4 Study Limitations and Future Directions

Most scientific research is limited with regards to time and budget constraints and therefore are not perfectly robust in terms of the processes followed. Instead, there is a balance to be achieved as researchers decide how best to allocate their resources in order to get as valid results as possible without sacrificing quality. As this study was a University Masters thesis there were budget and time constraints therefore sample numbers were restricted and resources that were readily available were used in order to minimise these factors. This thus impacted on some of the decisions made, such as which methods to use for ANA and anti-DFS70 detection. As this study took place in a clinical laboratory, their ANA and ENA methods and processes were used as this prevented further testing on patient samples and reduced both the time taken to test samples and the costs of the study. Since samples numbers were restricted this is likely to be the reason why some associations in this study did not reach statistical significance. Thus the findings of this study need to be further examined with a larger cohort of patients, however this study has provided a framework from which any future studies could expand upon.

Further studies should include a larger sample size for better statistical associations, and should also include additional ANA and anti-DFS70 detection assays from different manufacturers for comparison purposes. Also, follow up data is required in order to confirm whether or not patients with anti-DFS70 antibodies develop SARD in years to come. As this study used routine specimens sent for ANA testing, the samples were mostly from known SARD patients or those with a suspicion of SARD. Therefore the overall prevalence of anti-DFS70 antibodies in healthy individuals in New Zealand could not be determined. Therefore, future studies could also include healthy individuals. The inclusion of other disease categories may also be of benefit.

Nevertheless, as this study took place in a real world setting, the results obtained here are a likely to be an accurate reflection of the prevalence and implications of anti-DFS70 in a New Zealand hospital population and this study has also highlighted areas of concern in ANA testing in general.

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Appendix A: Ethics Approval

a) Health and Disability Ethics Approval Letter

Health and Disability Ethics Committees

Health and Disability Ethics Committees
Ministry of Health
Freyberg Building
20 Aitken Street
PO Box 5013
Wellington
6011

0800 4 ETHICS
hdecs@moh.govt.nz

05 August 2015

Mrs Stacey Lucas
29a Reimers Ave
Mt Eden / Auckland 1024

Dear Mrs Lucas

Re:	**Ethics ref:**	**15/CEN/103**
	Study title:	Incidence and clinical significance of anti-DFS70 in ANA positive patients undergoing routine ANA testing in a New Zealand public hospital.

I am pleased to advise that this application has been *approved* by the Central Health and Disability Ethics Committee. This decision was made through the HDEC-Full Review pathway.

Summary of Study

1. The Committee noted that this is an audit involving human tissue. The study will add one additional test that may be added to routine testing if it assists with diagnosis and the identification of false positives.

Summary of ethical issues (resolved)

The main ethical issues considered by the Committee and addressed by the Researcher are as follows.

1. Please store data generated from the study for 10 years as per health information privacy code.
2. The Committee requested that in future applications that answers about benefit (P.4.1) should include prevalence of Maori. It would be useful to talk about statistics that the research is covering and how it may improve Maori health. If there is no increased prevalence for Maori simply state this.
3. Similarly, questions about Maori cultural issues (P.4.2) you should include information on Maori and their value of human tissue.

Conditions of HDEC approval

HDEC approval for this study is subject to the following conditions being met prior to the commencement of the study in New Zealand. It is your responsibility, and that of the study's sponsor, to ensure that these conditions are met. No further review by the Central Health and Disability Ethics Committee is required.

Standard conditions:

1. Before the study commences at *any* locality in New Zealand, all relevant regulatory approvals must be obtained.

2. Before the study commences at *a given* locality in New Zealand, it must be authorised by that locality in Online Forms. Locality authorisation confirms that the locality is suitable for the safe and effective conduct of the study, and that local research governance issues have been addressed.

After HDEC review

Please refer to the *Standard Operating Procedures for Health and Disability Ethics Committees* (available on www.ethics.health.govt.nz) for HDEC requirements relating to amendments and other post-approval processes.

Your next progress report is due by 29 July 2016.

Participant access to ACC

The Central Health and Disability Ethics Committee is satisfied that your study is not a clinical trial that is to be conducted principally for the benefit of the manufacturer or distributor of the medicine or item being trialled. Participants injured as a result of treatment received as part of your study may therefore be eligible for publicly-funded compensation through the Accident Compensation Corporation (ACC).

Please don't hesitate to contact the HDEC secretariat for further information. We wish you all the best for your study.

Yours sincerely,

Mrs Helen Walker
Chairperson
Central Health and Disability Ethics Committee

Encl: appendix A: documents submitted
appendix B: statement of compliance and list of members

b) Waitemata District Health Board Ethics Committee Authorisation Report

Health and Disability Ethics Committees

Authorisation report

Study ref:	**15/CEN/103**
Study title:	Incidence and clinical significance of anti-DFS70 in a New Zealand population
Status:	Application decision given - Decision: decision of "approved" 30/07/2015 01:42:00

This authorisation report was generated by Waitemata District Health Board on 17 Aug 2015 at 11:49 AM

Authorisation Type	Authorisor	Date and time	Lead Investigator(s) at locality
Co-ordinating Investigator	Stacey Lucas	08 Jul 2015, 02:49 PM	Stacey Lucas
Other Investigator	Fabrice Merien	17 Aug 2015, 08:32 AM	
Primary Contact Person	Stacey Lucas	08 Jul 2015, 02:49 PM	Stacey Lucas
Locality	Waitemata DHB	17 Aug 2015, 11:46 AM	Stacey Lucas

Electronic Authorisations History

Date	Authorisation Type	Action
17 Aug 2015, 11:46 AM	Locality	Authorisation given by Waitemata District Health Board
17 Aug 2015, 11:38 AM	Locality	Request for authorisation accepted by Waitemata District Health Board
17 Aug 2015, 08:32 AM	Other Investigator	Authorisation given by Dr Fabrice Merien
17 Aug 2015, 08:24 AM	Other Investigator	Request for authorisation accepted by Dr Fabrice Merien
16 Aug 2015, 06:56 PM	Other Investigator	Request for authorisation sent by Mrs Stacey Lucas to Dr Fabrice Merien
16 Aug 2015, 06:50 PM	Locality	Request for authorisation sent by Mrs Stacey Lucas to Waitemata District Health Board
08 Jul 2015, 02:49 PM	Primary Contact Person	Authorisation given by Mrs Stacey Lucas
08 Jul 2015, 02:49 PM	Co-ordinating Investigator	Authorisation given by Mrs Stacey Lucas
08 Jul 2015, 02:35 PM	Co-ordinating Investigator	Authorisation invalidated by data change
07 Jul 2015, 02:15 PM	Co-ordinating Investigator	Authorisation given by Mrs Stacey Lucas

AM

c) Waitemata Distric Health Board Maori Review Approval Letter

From: Helen Wihongi (WDHB)
Sent: Thursday, 13 August 2015 1:37 p.m.
To: Stacey Lucas (WDHB)
Cc: Rose Smart (WDHB)
Subject: RE: Maori review

Tēnā koe Stacey,

Thank you for getting back to me. Please take this email to represent full approval for the study taking into account the recommendations made by the ethics committee for future studies.

Nga mii

Dr Helen Wihongi I **Research Advisor – Māori**
He Kamaka Waiora I **Waitemata and Auckland DHB**

Appendix B: Research Outputs

a) Sample Cohort Demographics

Sample	Age	Sex	Ethnicity	Ethnic Group
1	82	F	NZ pakeha	NZ E
2	17	F	NZ pakeha	NZ E
3	16	M	NZ pakeha	NZ E
4	69	F	NZ pakeha	NZ E
5	48	F	NZ pakeha	NZ E
6	25	F	NZ pakeha	NZ E
7	57	F	NZ pakeha	NZ E
8	41	F	NZ pakeha	NZ E
9	58	F	Japan/Asian	A
10	22	M	Asian	A
11	39	F	China/Chinese	A
12	71	M	Ireland/European	OE
13	19	M	Samoan	PI
14	36	F	NZ pakeha	NZ E
14	74	M	NZ pakeha	NZ E
15	42	M	NZ pakeha	NZ E
16	77	F	England/European	OE
17	33	F	Fiji/Indian	A
18	76	F	NZ pakeha	NZ E
19	54	F	Malaysia/Chinese	A
20	37	F	China/Chinese	A
21	70	M	NZ pakeha	NZ E
22	72	M	NZ pakeha	NZ E
23	25	M	NZ pakeha	NZ E
24	67	F	England/European	OE
25	60	F	NZ pakeha	NZ E
26	72	M	NZ pakeha	NZ E
27	54	F	NZ pakeha	NZ E
28	68	F	NZ pakeha	NZ E
29	70	F	Fiji/Indian	A
30	74	F	NZ pakeha	NZ E
31	47	M	NZ pakeha	NZ E
32	89	F	NZ pakeha	NZ E
33	67	M	Iran/Middle Eastern	ME
34	75	M	NZ pakeha	NZ E
35	73	F	China/Chinese	A
36	60	F	Korea/Asian	A
37	53	F	England/European	OE
38	88	F	NZ pakeha	NZ E
39	63	F	Samoan	PI
40	88	F	England/European	OE

Sample	Age	Sex	Ethnicity	Ethnic Group
41	87	F	England/European	OE
42	76	F	Denmark/European	OE
43	84	M	NZ pakeha	NZ E
44	77	F	Croatia	OE
45	74	F	NZ pakeha	NZ E
46	98	M	Hong Kong/Chinese	A
47	64	F	SA/European	OE
48	72	M	England/European	OE
49	45	F	NZ pakeha	NZ E
50	54	F	Samoan	PI
51	27	F	Korea/Asian	A
52	74	M	NZ pakeha	NZ E
53	13	F	England/European	OE
54	38	F	NZ pakeha	NZ E
55	43	F	NZ pakeha	NZ E
56	62	M	Fiji/Indian	A
57	47	M	Bangladesh/Indian	A
58	56	M	Fiji/Indian	A
59	76	F	NZ pakeha	NZ E
60	14	F	NZ pakeha	NZ E
61	50	F	Philippines/SE Asian	A
62	32	F	Kiribati/Pacific	PI
63	65	F	Fiji/Indian	A
64	71	F	NZ pakeha	NZ E
65	37	F	Indian	A
66	20	F	NZ pakeha	NZ E
67	24	F	Maori	Maori
68	75	F	GB/European	OE
69	79	F	NZ pakeha	NZ E
70	73	F	NZ pakeha	NZ E
71	60	F	NZ pakeha	NZ E
72	89	F	England/European	OE
73	72	M	Tuvalu/Pacific	PI
74	87	M	Fiji/Indian	A
75	74	M	NZ pakeha	NZ E
76	50	M	NZ pakeha	NZ E
77	79	F	England/European	OE
78	65	M	NZ pakeha	NZ E
79	49	F	NZ pakeha	NZ E
80	82	M	China/Chinese	A
81	65	F	NZ pakeha	NZ E
82	57	F	Singapore/SE Asian	A
83	54	F	SA/European	OE
84	25	M	Asian	A

Sample	Age	Sex	Ethnicity	Ethnic Group
85	65	F	Latin America	Other
86	19	F	NZ pakeha	NZ E
87	84	M	NZ pakeha	NZ E
88	67	M	England/European	OE
89	77	F	Sri Lanka/Indian	A
90	74	F	Russian	OE
91	66	F	NZ pakeha	NZ E
92	66	F	NZ pakeha	NZ E
93	90	F	Ireland/European	OE
94	88	F	GB/European	OE
95	71	F	England/European	OE
96	35	F	NZ pakeha	NZ E
97	88	F	NZ pakeha	NZ E
98	59	F	NZ pakeha	NZ E
99	28	F	Samoan	PI
100	89	F	NZ pakeha	NZ E
101	54	M	Samoan	PI
102	49	M	China/Chinese	A
103	32	F	Spain	Other
104	54	F	Tonga	PI
105	88	F	Italian / European	OE
106	60	F	NZ pakeha	NZ E
107	51	M	NZ pakeha	NZ E
108	72	F	Iraq/middle eastern	ME
109	73	F	NZ pakeha	NZ E
110	41	F	NZ pakeha	NZ E
111	44	F	China/Chinese	A
112	28	F	NZ pakeha	NZ E
113	65	F	NZ pakeha	NZ E
114	28	F	Samoan	PI
115	49	F	Ghana/African	Other
116	38	F	NZ pakeha	NZ E
117	43	F	China/Chinese	A
118	60	F	NZ pakeha	NZ E
119	54	M	SA/European	OE
120	53	M	Samoan	PI
121	40	F	England/European	OE
122	45	F	NZ pakeha	NZ E
123	32	F	NZ pakeha	NZ E
124	79	M	NZ pakeha	NZ E
125	29	M	China/Chinese	A
126	61	F	NZ pakeha	NZ E
127	57	M	NZ pakeha	NZ E
128	29	M	China/Chinese	A

Sample	Age	Sex	Ethnicity	Ethnic Group
129	37	M	NZ pakeha	NZ E
130	26	F	NZ pakeha	NZ E
131	45	F	Fiji/Indian	A
132	46	F	China/Chinese	A
133	60	F	NZ pakeha	NZ E
134	65	F	Samoan	PI
135	42	F	Maori	Maori
136	26	F	NZ pakeha	NZ E
137	39	F	Maori	Maori
138	26	F	NZ pakeha	NZ E
139	27	F	China/Chinese	A
140	62	F	Aus/European	OE
141	58	F	NZ pakeha	NZ E
142	50	F	NZ pakeha	NZ E
143	68	F	SA/European	OE
144	54	M	England/European	OE
145	74	F	Pakistan/Indian	A
146	42	F	NZ pakeha	NZ E
147	19	F	NZ pakeha	NZ E
148	19	F	Philippines/SE Asian	A
149	61	F	NZ pakeha	NZ E
150	54	M	SA/European	OE
151	59	F	China/Chinese	A
152	27	F	China/Chinese	A
152	59	F	Maori	Maori
153	66	F	NZ pakeha	NZ E
154	45	F	Fiji/Indian	A
155	31	F	NZ pakeha	NZ E
156	47	M	GB/European	OE
157	36	F	Aus/European	OE
158	73	F	NZ pakeha	NZ E
159	65	F	Samoan	PI
160	19	F	NZ pakeha	NZ E
161	57	F	NZ pakeha	NZ E
162	26	F	NZ pakeha	NZ E
163	60	F	Samoan	PI
164	65	F	NZ pakeha	NZ E
165	19	F	NZ pakeha	NZ E
166	74	F	NZ pakeha	NZ E
167	53	M	Samoan	PI
168	65	F	Samoan	PI
169	29	F	NZ pakeha	NZ E
170	47	M	Fiji/Fijian	PI
171	39	F	Tongan	PI

Sample	Age	Sex	Ethnicity	Ethnic Group
172	53	F	Korean/Asian	A
173	32	F	NZ pakeha	NZ E
174	18	F	Pacific	PI
175	74	F	NZ pakeha	NZ E
176	47	M	Fiji/Fijian	PI
177	32	F	NZ pakeha	NZ E
178	41	F	NZ pakeha	NZ E
179	67	F	Zim/European	OE
180	54	M	SA/European	OE
181	19	F	Zim/European	OE
182	26	F	NZ pakeha	NZ E
183	59	F	Maori	Maori
184	32	F	NZ pakeha	NZ E
185	26	F	NZ pakeha	NZ E
186	62	F	SA/European	OE
187	27	F	China/Chinese	A
188	47	M	Fiji/Fijian	PI
189	18	F	Pacific	PI
190	48	F	Fiji/Indian	A
191	50	F	China/Chinese	A
192	75	F	NZ pakeha	NZ E
193	64	F	NZ pakeha	NZ E
194	55	F	NZ pakeha	NZ E
195	27	F	NZ pakeha	NZ E
196	23	F	NZ pakeha	NZ E
197	23	F	NZ pakeha	NZ E
198	42	F	Maori	Maori
199	52	F	NZ pakeha	NZ E
200	31	F	NZ pakeha	NZ E
201	54	M	SA/European	NZ E
202	18	F	Tongan	PI
203	64	F	NZ pakeha	NZ E
204	40	F	Korean/Asian	A
205	62	F	Fiji/Indian	A
206	64	F	NZ pakeha	NZ E
207	22	F	NZ pakeha	NZ E
208	44	F	NZ pakeha	NZ E
209	48	F	NZ pakeha	NZ E

Note. F = Female; M = male; A = Asian; PI = Pacific Islander; NZ E = NZ European; OE = Other European

b) Cohort Results and Clinical Details

Sample	ANA IIF NOVA View (LIU)	ANA IIF final pattern	ANA ELISA (ANA#)	anti-DFS70 CIA (CU)	ENA ELISA (SSA,SSB,SM,Sm/RNP, Jo1,Scl70,dsDNA, centromere)	SARD/non-SARD	Diagnosis / clinical symptoms	ANA comments
1	centromere (232)	centromere, homogenous	P (5.2119)	P (33)	Centromere (82)	Non-SARD	Metastic lung cancer	History of anti-centromere antibodies
2	unrecognised (438)	speckled	p (2.7507)	P (>450)	Scl70(33)	Non-SARD	Recurrent blistering, going for biopsy, no other history	2013 - homogenous (1288), anti-Scl70
3	unrecognised (179)	homogenous	P (2.5357)	P (76)	N	Non-SARD	Mild pan ulcerative colitis, no other history	No previous ANA history
4	centromere (305)	centromere	P (2.9072)	P (262)	SSA(29), Centromere(38)	Non-SARD	Sudden loss of sensation, facial nerve distribution. Otherwise fit and well. No known autoimmune condition	No previous ANA history
5	N (12)	N	P (1.0561)	P (119)	N	Non-SARD	Primary Raynaud's, eosinophilic oesophagitis	2015 - homogenous (80), ENA negative
6	homogenous (215)	homogenous	P (3.2)	P (51.8)	dsDNA(>200)	Non-SARD	2x miscarriage, seizures ?false positive dsDNA as negative by FARR	Anti-cardiolipin, lupus anticoagulant, 2009 - speckled (160)
7	unrecognised (146)	other	P (1.0097)	P (414)	N	Non-SARD	Gastro problems	No previous ANA history
8	homogenous (215)	homogenous	P (1.9792)	P (137)	N	SARD	SLE	2012 - Homogenous (640), ENA negative

Sample	ANA IIF NOVA View (LIU)	ANA IIF final pattern	ANA ELISA (ANA#)	anti-DFS70 CIA (CU)	ENA ELISA (SSA,SSB,SM,Sm/RNP, Jo1,Scl70,dsDNA, centromere)	SARD/non-SARD	Diagnosis / clinical symptoms	ANA comments
9	unrecognised	homogenous	p	N	N	Non-SARD	Rash	History high titer centromere
10	N	N	P	N	SSB(22)	Non-SARD	Ulcerative proctitis	No previous ANA history
11	homogenous	homogenous	P	N	N	Non-SARD	Fibroids, stillbirth	No previous ANA history
12	homogenous	homogenous	P	N	N	Non-SARD	Idiopathic pulmonary fibrosis	No previous ANA history
13	homogenous	homogenous	P	N	N	Non-SARD	Idiopathic pulmonary hypertension, gout	Previous ANA negative
14	unrecognised	other	p	N	N	Non-SARD	Persistent dyspnoea and respiratory impairment	Previous ANA negative
14	speckled	speckled	P	N	Sm(24)	Non-SARD	Budd Chiari (liver)	Positive ANA for a few months
15	N	N	P	N	N	Non-SARD	Bowel disorder	No previous ANA history
16	homogenous	homogenous	N	N	N	Non-SARD	Hypertension/COPD	No previous ANA history
17	homogenous	homogenous	N	N	N	Non-SARD	Chronic kidney disease	Previous ANA negative
18	homogenous	homogenous	N	N	N	Non-SARD	Discoid lupus	Previous ANA negative
19	unrecognised	speckled	P	N	N	Non-SARD	Pneumonia, hypertension	No previous ANA history
20	N	N	P	N	N	Non-SARD	Most likely functional bowel disorder	No previous ANA history

Sample	ANA IIF NOVA View (LIU)	ANA IIF final pattern	ANA ELISA (ANA#)	anti-DFS70 CIA (CU)	ENA ELISA (SSA,SSB,SM,Sm/RNP, Jo1,Scl70,dsDNA, centromere)	SARD/non-SARD	Diagnosis / clinical symptoms	ANA comments
21	speckled	speckled	P	N	SSA(27)	Non-SARD	Pericarditis and pericardial effusion	Previous anti-SSA
22	Cytoplasmic	other	P	N	N	Non-SARD	Gout	No previous ANA history
23	N	N	P	N	N	Non-SARD	Unexplained deranged liver function tests	Previous speckled(80), ENA negative
24	homogenous	homogenous	P	N	N	Non-SARD	Crohn's colitis	Previous ANA negative
25	speckled	other	P	N	N	Non-SARD	Gynocological problems, deranged liver functions	2012 -homogenous (>1280), ENA negative
26	homogenous	homogenous	P	N	dsDNA(75)	Non-SARD	Coeliac	No previous ANA history
27	homogenous	homogenous	P	N	N	Non-SARD	Crohn's disease	No previous ANA history
28	homogenous	homogenous	P	N	N	Non-SARD	Respiratory problems	Prev homogenous (>1250), ENA negative
29	N	N	P	N	N	Non-SARD	SIADH	2015 - diffuse (320), ENA negative
30	unrecognised	other	P	N	N	Non-SARD	Stroke	No previous ANA history
31	speckled	speckled	P	N	N	Non-SARD	Psychosis	2004 - speckled
32	speckled	speckled	P	N	N	Non-SARD	Bowel problems	No previous ANA history
33	N	N	P	N	N	Non-SARD	Cardiology problems	No previous ANA history

Sample	ANA IIF NOVA View (LIU)	ANA IIF final pattern	ANA ELISA (ANA#)	anti-DFS70 CIA (CU)	ENA ELISA (SSA,SSB,SM,Sm/RNP, Jo1,Scl70,dsDNA, centromere)	SARD/non-SARD	Diagnosis / clinical symptoms	ANA comments
34	N	N	P	N	N	Non-SARD	Chronic Kidney disease, diabete mellitus	No previous ANA history
35	speckled	speckled	P	N	N	Non-SARD	Reflux	2009 – speckled, SSA, SSB
36	homogenous	homogenous	P	N	N	Non-SARD	Thyroid nodules	No previous ANA history
37	homogenous	homogenous	P	N	N	Non-SARD	Coeliac	2012 - ANA negative. 2014 - homogenous (320)
38	N	N	P	N	N	Non-SARD	Inflammatory polyarthritis	History of diffuse high titre ANA, ENA negative
39	unrecognised	speckled	P	N	N	Non-SARD	Raynaud's phenomenon	Previous nucleolar/speckled (1280), SSA
40	N	N	P	N	N	Non-SARD	Respiratory problems	2011 - Negative ANA
41	homogenous, cytoplasm positive	homogenous	P	N	N	Non-SARD	Hepatitis	No previous ANA history
42	homogenous	homogenous	P	N	N	Non-SARD	Lung disease	Previous negative ANA
43	N	N	P	N	N	Non-SARD	Bone pain	No previous ANA history
44	homogenous	homogenous, speckled	P	N	N	Non-SARD	Heart problems	No previous ANA history
45	unrecognised	homogenous	P	N	N	Non-SARD	Lung cancer	No previous ANA history

Sample	ANA IIF NOVA View (LIU)	ANA IIF final pattern	ANA ELISA (ANA#)	anti-DFS70 CIA (CU)	ENA ELISA (SSA,SSB,SM,Sm/RNP, Jo1,Scl70,dsDNA, centromere)	SARD/non-SARD	Diagnosis / clinical symptoms	ANA comments
46	N	N	P	N	N	Non-SARD	Heart problems	No previous ANA history
47	N	N	P	N	N	Non-SARD	Cardiac problems	Previous ANA negative
48	homogenous	homogenous	P	N	N	Non-SARD	interstitial lung disease, heart problems	2015 speckled (640), ENA negative
49	homogenous	homogenous	N	N	N	Non-SARD	No clinical details	No previous ANA history
50	unrecognised, cytoplasm positive	other	P	N	N	Non-SARD	Mildly abnormal liver function tests - ?non-alcoholic fatty liver	Previous ANA negative
51	speckled	speckled	P	N	N	Non-SARD	ANCA associated glomerulonephritis	2015 - homogenous/nucleolar, Sm, Sm/RNP
52	homogenous	homogenous	P	N	N	Non-SARD	Acute kidney injury, ischaemic heart disease	No previous ANA history
53	N	N	P	N	N	Non-SARD	Left knee pain, no signs arthritis yet	No previous ANA history
54	homogenous	homogenous	P	N	N	Non-SARD	Bechet’s disease	Previous homogenous(360), ENA negative
55	unrecognised	homogenous	P	N	N	Non-SARD	Pericardial effusion ? cause	Previous ANA negative
56	unrecognised	other	N	N	N	Non-SARD	Chronic kidney disease, diabetes mellitus, hypertension	No previous ANA history

Sample	ANA IIF NOVA View (LIU)	ANA IIF final pattern	ANA ELISA (ANA#)	anti-DFS70 CIA (CU)	ENA ELISA (SSA,SSB,SM,Sm/RNP, Jo1,Scl70,dsDNA, centromere)	SARD/non-SARD	Diagnosis / clinical symptoms	ANA comments
57	unrecognised	homogenous	P	N	N	Non-SARD	Joint stiffness ?low grade connective tissue disease	Previous homogenous (1280), dsDNA weak positive
58	homogenous	homogenous	N	N	N	Non-SARD	Small airways disease ?cause	
59	unrecognised	centromere	P	N	Centromere (89)	Non-SARD	Primary biliary cirrhosis	No previous ANA history
60	homogenous	homogenous	P	N	N	Non-SARD	Foetal valproate syndrome	Previous SSA, Sm
61	speckled	speckled	P	N	SSA(28)	Non-SARD	Hypothyroidism	Previous ANA negative
62	N	N	P	N	dsDNA(33)	Non-SARD	Persistent proteinuria	Previous ANA negative
63	unrecognised	homogenous	P	N	dsDNA(>200)	Non-SARD	Liver problems, diabetes mellitus	Previous ANA negative
64	unrecognised	Other	P	N	N	Non-SARD	Unexplained weight loss	No previous ANA history
65	speckled	speckled	P	N	N	Non-SARD	No other features of connective tissue disease	2015 - speckled (1280), ENA negative
66	speckled	speckled	P	N	N	Non-SARD	Irritable bowel syndrome	No previous ANA history
67	homogenous	homogenous	P	N	dsDNA(30)	Non-SARD	Raynaud’s phenomenon	Previous homogenous (160), dsDNA
68	speckled	speckled	P	N	SSB(159)	Non-SARD	Bone problems	History of anti-SSB
69	homogenous	homogenous	P	N	Scl70(27)	Non-SARD	Adenocarcinoma	No previous ANA history
70	centromere	centromere	P	N	Centromere (112)	Non-SARD	Primary biliary cirrhosis	No previous ANA history

Sample	ANA IIF NOVA View (LIU)	ANA IIF final pattern	ANA ELISA (ANA#)	anti-DFS70 CIA (CU)	ENA ELISA (SSA,SSB,SM,Sm/RNP, Jo1,Scl70,dsDNA, centromere)	SARD/non-SARD	Diagnosis / clinical symptoms	ANA comments
71	homogenous	homogenous, centromere	P	N	Centromere (81), SSB(51)	Non-SARD		Previous centromere, SSB
72	homogenous	homogenous	P	N	N	Non-SARD	Multifactorial anaemia, heart problems	Previous ENA negative
73	homogenous	homogenous	P	N	dsDNA(25)	Non-SARD	Chronic kidney disease	No previous ANA history
74	homogenous	homogenous	P	N	N	Non-SARD	Chronic and generalised itch	No previous ANA history
75	homogenous	homogenous	P	N	SSA(38)	Non-SARD	General tiredness	No previous ANA history
76	N	N	P	N	dsDNA(26)	Non-SARD	No significant connective tissue symptoms to suggest connective tissue disease to explain a positive ds-DNA, Partial Raynaud's phenomenon	Previous homogenous (80), dsDNA
77	speckled	Other	P	N	N	Non-SARD	Acute pericardial effusion	Previous nucleolar/speckled (1280), SSA
78	nucleolar	Nucleolar	P	N	N	Non-SARD	Adenocarcinoma	No previous ANA history
79	N	N	P	N	N	Non-SARD	Abnormal liver function tests	No previous ANA history
80	speckled	speckled	P	N	N	Non-SARD	End stage liver disease with cirrhosis	No previous ANA history

Sample	ANA IIF NOVA View (LIU)	ANA IIF final pattern	ANA ELISA (ANA#)	anti-DFS70 CIA (CU)	ENA ELISA (SSA,SSB,SM,Sm/RNP, Jo1,Scl70,dsDNA, centromere)	SARD/non-SARD	Diagnosis / clinical symptoms	ANA comments
81	speckled, cytoplasm positive	speckled	P	N	N	Non-SARD	Primary biliary cirrhosis	Previous speckled (80), ENA negative
82	homogenous	homogenous	P	N	SSA(40)	Non-SARD	Low grade undifferentiated connective tissue disease	Previous homogenous (80), SSA, dsDNA
83	N	N	P	N	N	Non-SARD	Polyarthralgia	2011 - ANA negative
84	unrecognised	speckled	P	N	Scl70 (37)	Non-SARD	Raynaud's Phenomenon	Previous homogenous (320), SSB, Scl70
85	homogenous	speckled	P	N	N	Non-SARD	Idiopathic pulmonary embolism, autoimmune haemolytic anaemia	Previous ANA negative
86	homogenous	homogenous	P	N	N	Non-SARD	Viral related rash	No previous ANA history
87	N	N	P	N	N	Non-SARD	Isolated acute severe thrombocytopenia ?drug related	No previous ANA history
88	unrecognised	other	N	N	N	Non-SARD	Idiopathic pulmonary fibrosis, deranged liver function tests	Previous speckled (80), ENA negative
89	speckled	speckled	P	N	dsDNA(33)	Non-SARD	Cirrhosis	No previous ANA history
90	speckled	speckled	P	N	SSA(82)	Non-SARD	History of photosensitivity and faint erythema , not SLE	Previous speckled (640), SSA

Sample	ANA IIF NOVA View (LIU)	ANA IIF final pattern	ANA ELISA (ANA#)	anti-DFS70 CIA (CU)	ENA ELISA (SSA,SSB,SM,Sm/RNP, Jo1,Scl70,dsDNA, centromere)	SARD/non-SARD	Diagnosis / clinical symptoms	ANA comments
91	N	N	P	N	N	Non-SARD	ITP, Chronic renal impairment	No previous ANA history
92	Nucleolar	Nucleolar	P	N	N	Non-SARD	Raynaud's Phenomenon	No previous ANA history
93	centromere	centromere	P	N	centromere(40)	Non-SARD	Haemoptysis, bronchiectasis	No previous ANA history
94	homogenous	homogenous	P	N	N	Non-SARD	Autoimmune hepatitis, polymyalgia rheumatica	Previous diffuse (1280), ENA negative
95	speckled	speckled	p	N	Scl70(156)	Non-SARD	?fibrotic organising pneumonia, no evidence of scleroderma	Previous homogenous (640), Scl70
96	homogenous	homogenous	P	N	SSA(92), Sm/RNP(21)	Non-SARD	?mixed connective tissue disease	Previous homogenous (1280), SSA, Sm/RNP
97	homogenous	homogenous	P	N	dsDNA(27)	Non-SARD	Vascular	No previous ANA history
98	N	N	P	N	N	Non-SARD	Acute hepatitis	No previous ANA history
99	homogenous	homogenous, speckled	p	N	Sm/RNP(88), dsDNA(59)	Non-SARD	Acute phospholipid syndrome, no symptoms of SLE at this stage	Previous speckled (80)
100	homogenous	homogenous	P	N	N	Non-SARD	Chronic kidney disease	No previous ANA history
101	speckled	speckled	P	N	N	Non-SARD	Raised lifer functions	2013 - speckled (160), ENA negative

Sample	ANA IIF NOVA View (LIU)	ANA IIF final pattern	ANA ELISA (ANA#)	anti-DFS70 CIA (CU)	ENA ELISA (SSA,SSB,SM,Sm/RNP, Jo1,Scl70,dsDNA, centromere)	SARD/non-SARD	Diagnosis / clinical symptoms	ANA comments
102	centromere	centromere	P	N	Centromere (75)	Non-SARD	Bilateral pneumonia, No classical symptoms suggestive of CREST	Previous centromere (320), ENA negative
103	speckled	speckled	P	N	Sm(48)	Non-SARD	Viral illness	No previous ANA history
104	speckled	speckled	P	N	SSA(27)	Non-SARD	?Irritable bowel syndrome	No previous ANA history
105	homogenous	homogenous	P	N	N	Non-SARD	Chronic kidney disease	No previous ANA history
106	cytoplasmic	other	P	N	N	Non-SARD	Raynaud's phenomenon	Previous ANA negative
107	homogenous + cytoplasm positive	homogenous	P	N	N	Non-SARD	Chronic Hepatitis C	Previous homogenous (80), ENA negative
108	homogenous	homogenous	P	N	dsDNA(30)	Non-SARD	Skin changes in the hands and dystrophic nails, not suggestive of Raynaud's	Previous ANA negative
109	homogenous	homogenous, speckled	P	N	N	Non-SARD	Arthritis, possible SjS - no diagnosis as yet	Previous homogenous/speckled (1280), Ro52
110	speckled	speckled	P	N	N	SARD	DM / PM	
111	unrecognised	speckled	P	N	SSA(168)	SARD	SjS	
112	speckled	speckled	P	N	SSA(168), SSB(65)	SARD	SLE / SjS	
113	homogenous	homogenous	P	N	SSA(156)	SARD	SjS	

Sample	ANA IIF NOVA View (LIU)	ANA IIF final pattern	ANA ELISA (ANA#)	anti-DFS70 CIA (CU)	ENA ELISA (SSA,SSB,SM,Sm/RNP, Jo1,Scl70,dsDNA, centromere)	SARD/non-SARD	Diagnosis / clinical symptoms	ANA comments
114	homogenous	speckled	P	N	Sm/RNP(38), dsDNA(29)	SARD	SLE / chronic kidney disease	
115	speckled	speckled	P	N	SSA(192), Sm(24)	SARD	Primary SjS with Raynaud's	
116	unrecognised	homogenous	N	N	dsDNA(51)	SARD	RA	
117	speckled	speckled	P	N	SSA(172)	SARD	Primary SjS	
118	unrecognised	speckled	P	N	SSA(>200), SSB(156), CEN(26)	SARD	SjS	
119	homogenous	homogenous	P	N	Sm(143), Sm/RNP(51), dsDNA(137)	SARD	SLE	
120	homogenous	homogenous	P	N	dsDNA(121)	SARD	SLE	
121	homogenous	homogenous	P	N	N	SARD	RA	
122	speckled	speckled	P	N	SSB(70)	SARD	RA	
123	speckled	speckled	P	N	SSA(>200), SSB(112)	SARD	SLE, secondary SjS	
124	homogenous	homogenous	P	N	N	SARD	SLE	
125	speckled	speckled	P	N	Sm/RNP (36)	SARD	SLE	
126	Nucleolar	Nucleolar	P	N	N	SARD	RA	
127	speckled	speckled	P	N	SSA(>200) SSB(>200)	SARD	Primary SjS	
128	speckled	speckled	P	N	Sm/RNP(39)	SARD	SLE	

Sample	ANA IIF NOVA View (LIU)	ANA IIF final pattern	ANA ELISA (ANA#)	anti-DFS70 CIA (CU)	ENA ELISA (SSA,SSB,SM,Sm/RNP, Jo1,Scl70,dsDNA, centromere)	SARD/non-SARD	Diagnosis / clinical symptoms	ANA comments
129	unrecognised	homogenous	P	N	N	SARD	Crest syndrome (SSc)	
130	speckled	speckled	P	N	Scl70(46), dsDNA(>200)	SARD	SLE	
131	speckled	speckled	P	N	SSA(>200), SSB(163)	SARD	SLE, secondary SjS	
132	speckled	speckled	P	N	SSA(>200), Sm(>200), Sm/RNP(>200), dsDNA(>200)	SARD	SLE, Raynaud's	
133	speckled	speckled	P	N	SSA(>200), SSB(182)	SARD	SjS	
134	speckled	speckled	P	N	Sm/RNP(22)	SARD	SLE, Raynaud's	
135	unrecognised	speckled	P	N	dsDNA(>200)	SARD	SLE	
136	speckled	speckled, homogenous	P	N	Sm(162), Sm/RNP(>200) Scl70(31), dsDNA(>200)	SARD	SLE	
137	homogenous	homogenous	P	N	dsDNA(165)	SARD	SLE	
138	unrecognised	speckled	P	N	Sm(188), Sm/RNP(>200), Scl70(35), dsDNA(>200)	SARD	SLE	
139	homogenous	homogenous	P	N	SSA(139), dsDNA(24)	SARD	SLE	
140	homogenous	homogenous	P	N	SSB(64), Scl70(52), dsDNA(35)	SARD	SLE	
141	speckled	speckled	P	N	Sm/RNP(22)	SARD	SLE	

Sample	ANA IIF NOVA View (LIU)	ANA IIF final pattern	ANA ELISA (ANA#)	anti-DFS70 CIA (CU)	ENA ELISA (SSA,SSB,SM,Sm/RNP, Jo1,Scl70,dsDNA, centromere)	SARD/non-SARD	Diagnosis / clinical symptoms	ANA comments
142	N	N	P	N	N	SARD	SLE, secondary SjS	
143	speckled	speckled	P	N	SSA(>200), SSB(20)	SARD	SjS	
144	speckled	speckled	p	N	Sm(20)	SARD	SLE	
145	unrecognised	speckled	P	N	SSA(146), SSB(125)	SARD	SLE	
146	unrecognised	speckled	P	N	SSA(>200), SSB(115)	SARD	SLE, secondary SjS	
147	speckled	speckled	P	N	SSA(73), SSB(171)	SARD	SLE, secondary SjS	
148	homogenous	homogenous	P	N	Sm(41), Sm/RNP(47), dsDNA(75)	SARD	SLE	
149	speckled	speckled	P	N	SSA(135), SSB(141)	SARD	SjS	
150	speckled	speckled	P	N	Sm(212), Sm/RNP(8), DsDNA(74)	SARD	SLE	
151	homogenous, speckled	homogenous, speckled	P	N	SSA(86), Sm (>200), Sm/RNP(174), dsDNA(44)	SARD	SLE	
152	homogenous	homogenous	P	N	SSA(112), Sm/RNP(184), dsDNA(54)	SARD	SLE	
152	centromere	centromere	p	N	Centromere(49)	SARD	Seronegative RA	
153	centromere	centromere	P	N	Centromere(230)	SARD	SSc	
154	speckled	speckled	P	N	SSA, SSB >200	SARD	SLE, secondary SjS	

Sample	ANA IIF NOVA View (LIU)	ANA IIF final pattern	ANA ELISA (ANA#)	anti-DFS70 CIA (CU)	ENA ELISA (SSA,SSB,SM,Sm/RNP, Jo1,Scl70,dsDNA, centromere)	SARD/non-SARD	Diagnosis / clinical symptoms	ANA comments
155	speckled	speckled	P	N	SSA(>200), SSB(83)	SARD	SLE, secondary SjS	
156	speckled	speckled	P	N	SSA(>200), SSB(206)	SARD	SjS with mild Raynaud's	
157	unrecognised	speckled	P	N	SSA, SSB(>200)	SARD	SjS	
158	speckled	speckled	P	N	SSA(>200), SSB(188)	SARD	SjS	
159	speckled	speckled	P	N	Sm(48), Sm/RNP(>200)	SARD	SLE	
160	N	N	P	N	N	SARD	RA	
161	homogenous	homogenous	P	N	N	SARD	SLE	
162	speckled	homogenous, speckled	P	N	Sm(>200), Sm/RNP(>200), Scl70(27) DsDNA(>200)	SARD	SLE	
163	speckled	speckled	P	N	Sm(>200), Sm/RNP(>200), dsDNA(>200)	SARD	SLE, Raynaud's phenomenon	
164	centromere	centromere	P	N	centromere(>200)	SARD	SLE	
165	homogenous	homogenous	P	N	SSA(60), Sm(30), Sm/RNP(75), dsDNA(182)	SARD	SLE	
166	speckled	speckled	P	N	SSA(>200) SSB(243)	SARD	SLE	

Sample	ANA IIF NOVA View (LIU)	ANA IIF final pattern	ANA ELISA (ANA#)	anti-DFS70 CIA (CU)	ENA ELISA (SSA,SSB,SM,Sm/RNP, Jo1,Scl70,dsDNA, centromere)	SARD/non-SARD	Diagnosis / clinical symptoms	ANA comments
167	homogenous, cytoplasm positive	homogenous	P	N	dsDNA >200	SARD	SLE	
168	speckled	speckled	P	N	Sm(55), Sm/RNP(>200)	SARD	SLE, Raynaud's phenomenon	
169	speckled	speckled	P	N	SSA(116), Sm/RNP(176), dsDNA(50)	SARD	SLE	
170	speckled	speckled	P	N	Sm(26), dsDNA(132)	SARD	SLE	
171	N	N	P	N	dsDNA (109)	SARD	SLE	
172	homogenous	homogenous	P	N	SSA(99), dsDNA(>200)	SARD	SLE	
173	homogenous	homogenous	P	N	dsDNA(>200)	SARD	SLE	
174	speckled	speckled	P	N	SSA(97), Sm(151), Sm/RNP(>200), Scl70(58), dsDNA(>200)	SARD	SLE	
175	speckled	speckled	P	N	SSA(156), SSB(126)	SARD	SLE	
176	homogenous	homogenous	P	N	dsDNA(170)	SARD	SLE	
177	speckled	speckled	p	N	SSA(>200) SSB(99)	SARD	SLE	
178	speckled	speckled	P	N	SSA(42), SSB(29)	SARD	SLE	

Sample	ANA IIF NOVA View (LIU)	ANA IIF final pattern	ANA ELISA (ANA#)	anti-DFS70 CIA (CU)	ENA ELISA (SSA,SSB,SM,Sm/RNP, Jo1,Scl70,dsDNA, centromere)	SARD/non-SARD	Diagnosis / clinical symptoms	ANA comments
179	homogenous	homogenous	P	N	N	SARD	SLE, Raynaud's phenomenon	
180	speckled	speckled	P	N	Sm(132), Sm/RNP(60.5), dsDNA (142)	SARD	SLE	
181	homogenous	homogenous	P	N	N	SARD	SLE	
182	homogenous	speckled	P	N	SSA(>200)	SARD	SLE	
183	speckled	speckled	P	N	SSA(72)	SARD	SLE	
184	speckled	speckled	P	N	SSA(>200), SSB(103)	SARD	SLE	
185	speckled	speckled	P	N	Sm(183), SmRNP(>200), Scl70(30), dsDNA(>200)	SARD	SLE	
186	unrecognised	speckled	P	N	Sm/RNP(82)	SARD	SLE	
187	homogenous	homogenous	P	N	SSA(88), Sm(>200), Sm/RNP(238), dsDNA(61)	SARD	SLE	
188	homogenous	homogenous	P	N	dsDNA(72)	SARD	SLE	
189	speckled	speckled	P	N	SSA(84), Sm(70), Sm/RNP(223), Scl70 (56), dsDNA(>200)	SARD	SLE	
190	speckled	speckled	p	N	N	SARD	RA	

Sample	ANA IIF NOVA View (LIU)	ANA IIF final pattern	ANA ELISA (ANA#)	anti-DFS70 CIA (CU)	ENA ELISA (SSA,SSB,SM,Sm/RNP, Jo1,Scl70,dsDNA, centromere)	SARD/non-SARD	Diagnosis / clinical symptoms	ANA comments
191	homogenous, cytoplasm positive	speckled	P	N	SSA(>200), SSB(152)	SARD	SLE	
192	homogenous	homogenous	P	N	N	SARD	RA	
193	homogenous	homogenous	P	N	SSA(158), SSB(124), dsDNA(>200)	SARD	Primary SjS / SLE.	
194	centromere	centromere	P	N	centromere(68)	SARD	RA	
195	speckled	speckled	P	N	Sm(132), Sm/RNP(>200), dsDNA (>200)	SARD	SLE	
196	homogenous	homogenous	P	N	dsDNA(>200)	SARD	SLE	
197	homogenous	homogenous	P	N	dsDNA(>200)	SARD	SLE	
198	homogenous	homogenous	P	N	dsDNA(42)	SARD	SLE	
199	speckled	speckled	P	N	SSA(168)	SARD	SLE	
200	speckled	speckled	P	N	SSA(266), SSB(105)	SARD	SLE	
201	speckled	speckled	P	N	Sm(146), Sm/RNP(61), dsDNA(64)	SARD	SLE	

Sample	ANA IIF NOVA View (LIU)	ANA IIF final pattern	ANA ELISA (ANA#)	anti-DFS70 CIA (CU)	ENA ELISA (SSA,SSB,SM,Sm/RNP, Jo1,Scl70,dsDNA, centromere)	SARD/non-SARD	Diagnosis / clinical symptoms	ANA comments
202	speckled, cytoplasm positive	speckled	P	N	SSA(204), Sm(77), Sm/RNP(97), dsDNA(>200)	SARD	SLE	
203	homogenous	homogenous	P	N	SSA(171), SSB(138), dsDNA(>200)	SARD	Primary SjS / SLE.	
204	homogenous	homogenous	P	N	Sm(30), Sm/RNP(54), dsDNA(60)	SARD	SLE, antiphospholipid syndrome	
205	speckled	speckled	P	N	SSB(30)	SARD	RA	
206	homogenous	speckled	P	N	SSA(226), SSB(195)	SARD	SjS	
207	speckled	speckled	P	N	N	SARD	SLE	
208	homogenous	homogenous	P	N	Scl70(134)	SARD	SSc	
209	homogenous	homogenous	P	N	Sm/RNP(190), dsDNA(>200)	SARD	SjS	
RCPA QAP1	unrecognised		P(1.44)	P(142)				
RCPA QAP2	unrecognised		P(1.27)	P(127)				

Note. P = positive; N = negative; SARD = systemic autoimmune rheumatic disease; RA = rheumatoid arthritis; SjS = Sjorgren's syndrome ; SSc = Systemic Sclerosis ; DM/PM = dermatomyositis/polymyositis; SLE = systemic lupus erythematosus; LIU = light intensity units; ANA = antinuclear antibody; ELISA = enzyme linked immunosorbent assay; ENA = extractable nuclear antigen; IIF = indirect immunofluorescence; RCPAQAP = Royal College of Pathologists of Australia Quality Assurance Program.