Prediction of development of immune mediated inflammatory diseases with antinuclear antibodies
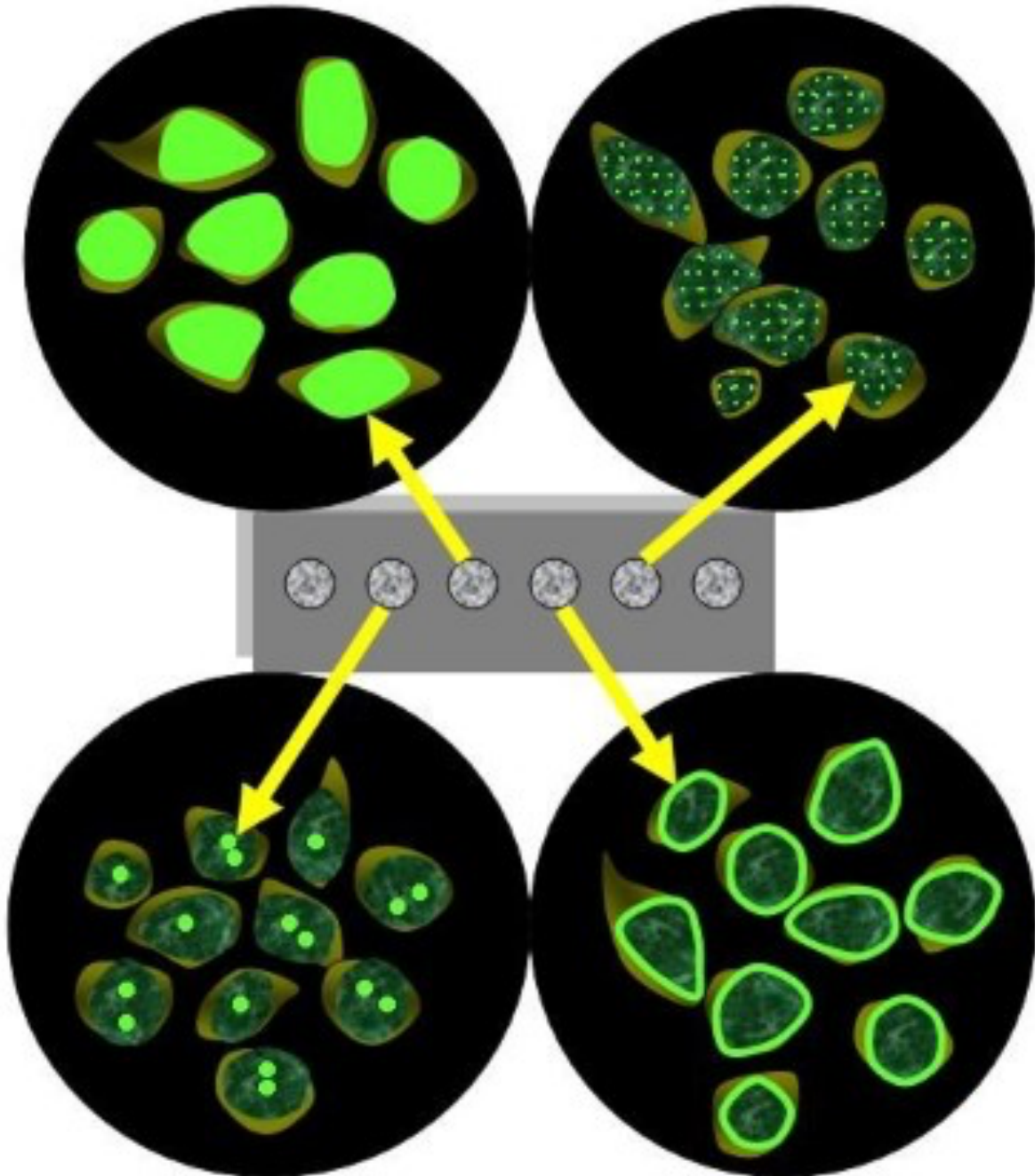
Author: Hélène van den Heuvel
Examiner: Maarten Limper
Supervisor: Tammo Brunekreef
Infection and Immunity, Utrecht University
22nd of March, 2022

Cover image: diagram of nuclear patterns commonly observed with a HEp2 screening test under a fluorescence microscope[1].

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Abbreviations

ANA: antinuclear antibody
APS: antiphospholipid syndrome
CLE: cutaneous lupus erythematosus
CTD: connective tissue disorder
IMID: immune mediated inflammatory disease
JIA: juvenile idiopathic arthritis
LLD: lupus like disease.
MCTD: mixed connective tissue disease
PM: polymyositis
PMDM: polymyositis and dermatomyositis
PMR: polymyalgia rheumatica
pSS: primary Sjögren's
RA: rheumatoid arthritis
SLE: systemic lupus erythematosus
SSc: systemic sclerosis

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Abstract

Immune mediated inflammatory diseases (IMIDs) are inflammatory conditions of which the driving molecular mechanisms currently remain unknown[2,3]. Treatment options include corticosteroids, immunosuppressants, and biologicals, none of which are curable options[5]. In order to prevent excessive tissue damage due to IMIDs, early diagnostics is crucial. This remains a challenge, as IMIDs are complex conditions and clinical manifestations vary largely between patients[6]. Antinuclear antibodies (ANAs) are autoantibodies that target nuclear molecules. Some ANA subtypes are exclusively associated with particular IMIDs and have shown to appear in the years prior to clinical onset of IMIDs[9,10,11]. Hence, ANAs have been proposed as potential predictors of future IMID development. The aim of the current study is to evaluate the predictive value of positive ANA screening tests and specific ANA tests for IMID development over the course of six years. The cohort of the study conducted by Otten and colleagues[8] in 2014 was used for this purpose. In their study, a total of 1030 patients for which ANA tests were requested were included. For the purpose of the current study, the electronic health records of these patients were analyzed using a text mining algorithm[12] to detect new diagnoses that have been established between 2014 and 2020. Based on these data, the predictive value of the ANA test results for future IMID development was evaluated. It was established that only a small proportion of the patients that were at high risk of developing an IMID, developed an IMID within six years after the ANA tests were conducted. Nonetheless, patients that developed an IMID were more likely to have positive ANA test results at baseline compared to patients with the same pretest probability for IMID development that did not develop an IMID. It is therefore proposed that ANA test results could be used as predictors for IMID development in the near future. As ANA test results should always be put in the context of the patient's clinical presentation, the ANA test results could be used as predictors for future IMID development in patients that have a high pretest probability of developing an IMID based on clinical guidance.

Introduction

Immune mediated inflammatory diseases (IMIDs) are distinct conditions that are caused by dysregulations of the immune system[2]. Even though the molecular mechanisms driving these IMIDs remain unknown, research has demonstrated that an imbalance of inflammatory cytokines could play a pivotal role in the pathogenesis of IMIDs[3]. In the developed world, the incidence of IMIDs is approximately 5-7%. The most common IMIDs include inflammatory bowl disease, multiple sclerosis, systemic lupus erythematosus (SLE), type 1 diabetes, rheumatoid arthritis (RA), and psoriasis[4]. Most IMIDs can be treated using corticosteroids, immunosuppressants, and biologicals. These treatment options reduce inflammation, prevent excessive tissue damage, and could possibly induce remission of disease. However, no curable treatment options are currently available[5].

As IMIDs are often chronic and progressive, early diagnosis is crucial for the prevention of tissue damage. Nonetheless, diagnostics of IMIDs remains a challenge, as not one test can be used to establish a particular diagnosis. Instead, clinicians use classification criteria in which clinical symptoms are combined with laboratory test results to confirm a diagnosis. This procedure is rather limited, as the clinical manifestations vary largely between patients. This makes IMIDs complex to define and diagnose[6].

Antinuclear antibodies (ANAs) could potentially play a role in facilitating improved diagnostics of IMIDs. ANAs are defined as autoantibodies that target nuclear molecules[7] such as dsDNA, small nuclear ribonucleoprotein, and centromeres[1]. Some ANAs are known to occur naturally in healthy individuals, but some ANAs are almost exclusively associated with particular IMIDs. As a result, the presence of these ANAs could be highly relevant in diagnostics of patients with suspected IMIDs[7]. In the University Medical Center Utrecht it is therefore the common procedure to test for the presence of ANAs in patients that are suspected of having an IMID. This is a two-step process consisting of an ANA screening test, known as the HEp2 screening, to confirm the presence of ANAs, and a subsequent specific test to determine the ANA subtypes that are present. The latter of these tests is known as a lineblot.

The main problem surrounding ANA tests is that the majority of the patients for which ANA tests are requested has a low pretest probability for a particular IMID. As a result, these ANA tests are often solely used for the purpose of screening rather than confirmation of the suspected condition. This poses a problem, as ANA tests may yield unexpected positive results for ANAs that do not fit with the clinical presentation of the patient. This may cause confusion regarding interpretation of the results, ultimately hampering the diagnostic process. In addition, specific ANA tests are often conducted to detect ANAs that are not compatible with the clinical manifestations, making this a very cost-ineffective method.

The current strategy surrounding ANA tests for the confirmation of IMIDs was critically addressed by Otten and colleagues[8]. In their study, they aimed to evaluate the clinical and financial efficacy of the current strategy between different commercial tests in a large cohort of unselected patients. They gathered data of a total of 1030 patients for which ANA tests were requested. Based on clinical presentation, the patients were divided into five categories distinguished by the pretest probability for an IMID: patients with established classification criteria for an IMID; patients with at least one symptom that is relatively common with an IMID; patients with at least one symptom that is a rare presenting symptom of an IMID; patients with unspecific symptoms or patients that were tested to exclude an IMID; patients with a suspicion of autoimmune hepatitis. For all patients an ANA screening test was performed, which could be a HEp2 screening test, a connective tissue disorder (CTD) screen, or both. Upon a positive ANA screening test result, a specific ANA test was performed to determine the types of ANAs present. In case of a positive HEp2 screening, a lineblot was performed and in case of a positive CTD screen, a CTD single analyte was performed (figure 1).
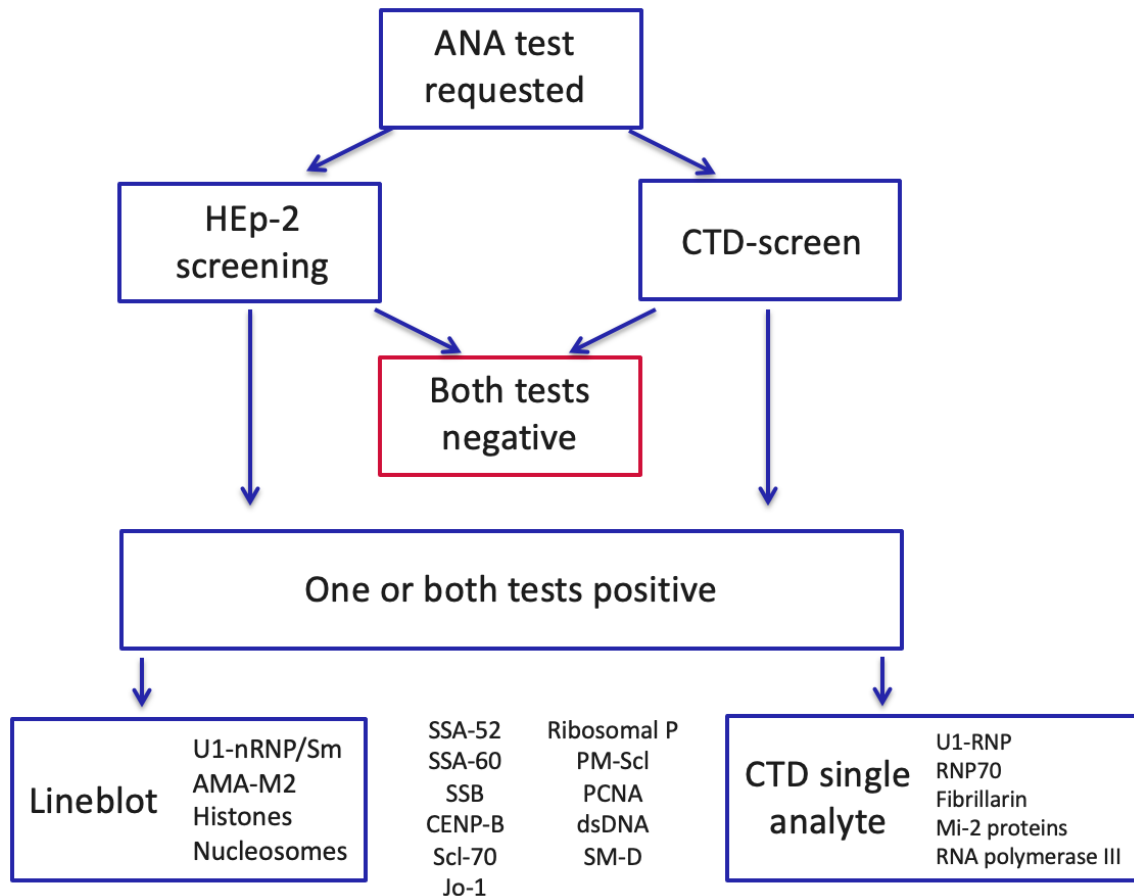
Figure 1: Study design of article by Otten et al.: Measurement of antinuclear antibodies and their fine specificities: time for a change in strategy? The HEp2 screening and CTD screen are both screening tests used to confirm the presence of ANAs. Once confirmed, a specific ANA test is performed. The main difference between the two specific ANA tests is the antigens present on the test, and therefore the types of ANAs that can be detected. The antigens presented in the boxes are solely present in the corresponding specific ANA test, whereas the antigens present in between are present on both specific ANA tests[8].

Interestingly, the authors recognized that many patients with a low pretest probability for an IMID were tested positive for particular ANAs. Likewise, patients with an established diagnosis tested positive for ANAs that were not associated with their diagnosis. It was found that in 83% of the cases specific ANA tests were conducted to find ANAs that did not fit the clinical presentation of the patient. In these cases, unexpected positive results can cause confusion regarding interpretation. As such, the authors proposed that ANA tests should only be conducted according to the clinical presentation of the patient. Consequently, ANA tests should no longer be conducted in patients with a low pretest probability of an IMID. This approach would be a cost-saving strategy that omits tests irrelevant for diagnostics. In addition, this approach limits generation of confusion due to unexpected positive ANA test results.

In the current study, patients from category 2, patients with at least one symptom that is a rare presenting symptom of an IMID, were more closely analyzed. This is a particularly special group of patients, as they had at least one symptom rarely associated with an IMID and no established diagnosis, but many of the patients had positive ANA test results. This is of interest as many previous studies have demonstrated ANAs can be present years before the onset of particular IMIDs. For instance, a study conducted in 1992 performed HEp2 screening tests on stored sera from individuals that were known to have develop SLE or mixed connective tissue disease (MCTD) in the future. The researchers found ANAs were more often present in sera from individuals that would develop SLE or MCTD at a later stage. It was therefore concluded that the appearance of ANAs often precedes clinical onset of SLE and MCTD[9]. In addition, a review published in 2017 showed that autoantibodies can be

present up to 13 years before clinical onset of RA and up to 8 years before clinical presentation of SLE[10].

In a different study, serum samples from 130 individuals were evaluated for the presence of ANAs in the years prior to clinical onset of SLE. Based on their results, it was concluded that ANAs appear in a large proportion of future SLE patients in the years before clinical onset. Some ANAs, such as anti-Ro and anti-La antibodies were found to appear in over half of the patients 5 years prior to diagnosis. Other ANAs such as anti-Sm and anti-nRNP antibodies were found to appear in a large proportion of the future SLE patients only months before clinical diagnosis (fig. 2). Even though these results indicate ANAs are present in a large proportion of the future SLE patients in the years before clinical onset of SLE, these results do not indicate that the presence of particular ANAs can predict future SLE development. In order to determine the predictive value of ANAs, a comparison of these results to data obtained from healthy individuals is required. For instance, anti-Ro and anti-La antibodies are known to be relatively common in healthy individuals, hence the presence of these ANAs cannot solely be used to predict future SLE development. On the other hand, the proportion of patients with anti-Sm and anti-nRNP antibodies strongly increases right before clinical diagnosis of SLE. Consequently, many patients gain these ANAs right before diagnosis and around the time clinical manifestations are likely to occur. As such, these antibodies could potentially be interesting as predictors of SLE in the near future[11].
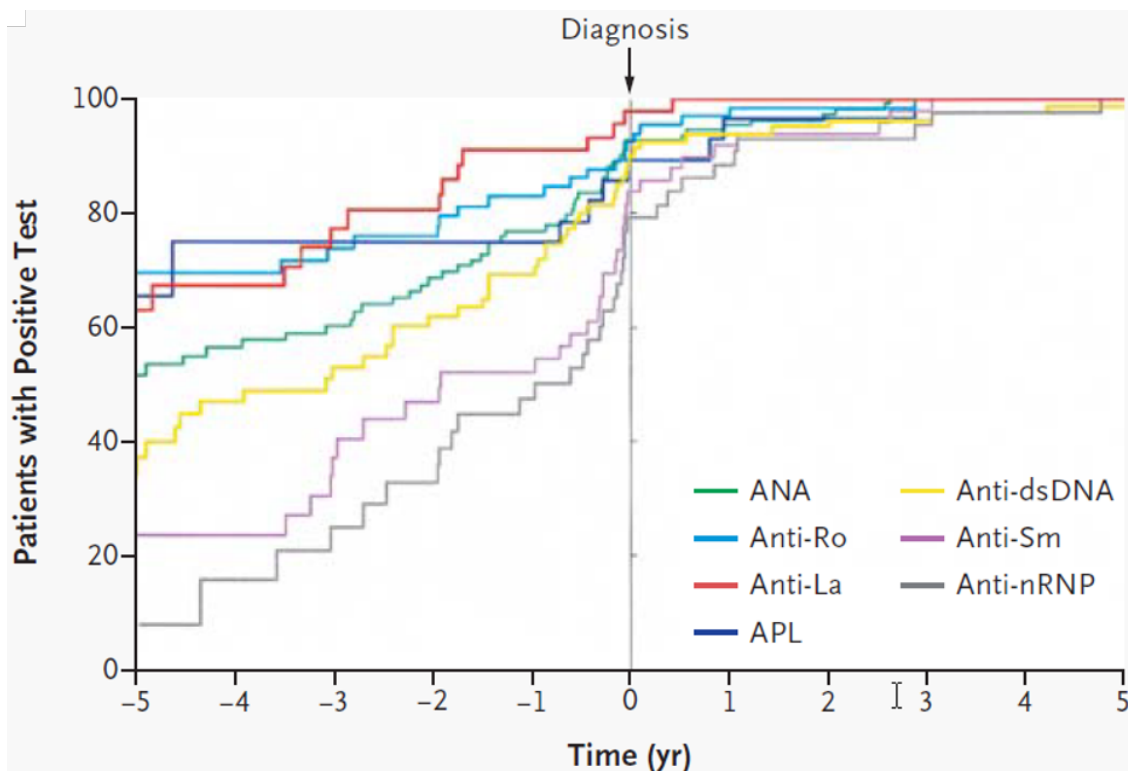

Figure 2: Kaplan-Meier curve showing the proportion of patients with positive ANA test results relative to the sample population (y-axis) to the time of diagnosis (x-axis). The total sample population consisted of 130 individuals that would develop SLE in the future. In the years prior to the diagnosis, these patients were tested for the presence of ANAs. It was found that in a large proportion of the future patients, ANAs are present in the years prior to diagnosis. The proportions of patients tested positive for particular autoantibodies at a certain time are indicated by the different colored lines[11].

Based on previous literature, it has become clear ANAs associated with IMIDs are present years before clinical onset of the disease. This raises the question whether the presence of ANAs could have predictive value for future IMID development and could therefore be relevant for early detection of IMIDs patients. The aim of the current study is to evaluate the predictive value of positive ANA screening tests and specific ANA tests for IMID development over the course of six years.

Methods

In the current study, the cohort of the 2014 study conducted by Otten and colleagues[8] was reanalyzed using a text mining algorithm[12]. This algorithm was used to find newly described diagnoses in the electronic healthy records of the patients recruited in the study. New diagnoses were manually evaluated for confirmation. Based on the results, the cohort was divided into four new categories: A) patients with a new diagnosis; B) patients with an established diagnosis at baseline or diagnosed around baseline with the ANA test results; C) patients with at least one symptom that is relatively common with an IMID at baseline but no current diagnosis; D) remaining patients that have no current diagnosis and did not have symptoms that are strongly associated with a particular IMID at baseline. In addition, for a total of 65 patients, additional ANA test results were obtained from ANA tests that were performed in the six years after the initial ANA test was performed.

Using the chi-square statistic, the ANA test results were compared between groups A, B, and C. The ANA screening test results and specific ANA test results were considered separately to determine whether the frequency of positive ANA test results differs between future patients, patients with an established diagnosis, and patients with symptoms but no diagnosis. Based on these data, the potential of ANA tests as predictors for future IMID development was evaluated.

In the analyses of the specific ANA tests, the compatibility of the positive ANA test results with the future-, established-, or suspected diagnosis was determined in groups A, B, and C respectively. Patients that had a suspected-, future-, or an established diagnosis that is not compatible with one or more ANAs were excluded from the analyses. This exclusion was performed to avoid an underestimation of the proportion of patients that tested positive for at least one ANA associated with the suspected-, future-, or established diagnosis. Examples of such patients include patients with suspected- or established RA or JIA.

Results

In this study, the cohort from the study conducted by Otten and colleagues[8] was used. Based on newly acquired information concerning IMID diagnoses, the cohort was divided into four new categories (Table 1).

Table 1: Summary of the cohort composition. The cohort was divided into four different groups based on their IMID status. The table summarizes the number of patients included in each group, the mean age within each group, and the male to female ratio.

	Number of patients	Mean age (years ± SD)	Proportion male (%)	Proportion female (5)
A: Patients with a new diagnosis	19*	40.8 (±17.0)	0	100
B: Patients with an established diagnosis at baseline or diagnosed around baseline with the ANA test results	258	42.6 (±22.0)	25.6	74.4
C: Patients with at least one symptom that is relatively common with an IMID at baseline but no current diagnosis	326	39.5 (±20.3)	37.1	62.9
D: remaining patients	427	41.0 (±20.9)	50.1	49.9
Total	1030	41.0 (±20.9)	38.9	61.1

*Only 19 out of a total of 1030 patients were diagnosed with a new IMID within 6 years after the initial ANA tests were conducted. This is a very small group to consider for statistical analysis.

In all groups, the mean age is around 40 years old with a standard deviation around 20. This illustrates that the ages vary greatly within the groups and are not remarkably different between the groups. Interestingly, all future patients were female. Also, the patients with an established diagnosis at baseline were predominantly female. Only in the group consisting of the remaining patients that did not have an established diagnosis or symptoms associated with an IMID, the male to female ratio was approximately 50%. This observation is of interest, as IMIDs are known to occur primarily in females. It is therefore no surprise that the future patients and patients with an established diagnosis are predominantly female. It is, however, remarkable that also the patients with at least one symptom commonly associated with an IMID but no current diagnosis were predominantly female.

In this research, the group of patients with a new diagnosis, group A, is of particular interest. Their characteristics provide clues for potential predictors of future IMID development. Based on evaluation of the electronic healthy records, the pre-test clinical consensus, current diagnosis, and the time between the initial ANA test and the diagnosis were established (Table 2).

Table 2: Summary of group A: Patients with a new diagnosis. The age, pre-test clinical consensus, current diagnosis, and the time between the initial ANA test and the diagnosis are summarized. PMDM = polymyositis and dermatomyositis; APS = antiphospholipid syndrome; SLE = systemic lupus erythematosus; JIA = juvenile idiopathic arthritis; MCTD = mixed connective disease; SSc = systemic sclerosis; RA = rheumatoid arthritis; pSS = primary Sjögren's; PM = polymyositis; CLE = cutaneous lupus erythematosus; PMR = polymyalgia rheumatica; LLD = lupus like disease.

*In four cases, the current diagnoses were additional to the one already present.

Age	Pre-test clinical consensus	Current diagnosis	Time between ANA test and diagnosis (days)
22	Serositis	MCTD	128
50	Raynaud	SSc	322
45	PMDM	PMDM and SSc*	902
57	Liver function disorder	RA	239
31	APS	SLE*	1776
68	Sicca	pSS/SSc	604
25	Cytopenia	SLE	1980
38	Arthralgia/myalgia	RA	610
53	Skin abnormalities compatible with SLE	PM and CLE	52
47	Sicca	SLE	362
29	APS	SLE and APS*	1127
59	Arthralgia/myalgia	PMR	289
68	Sicca	pSS	1371
61	Interstitial lung disease	RA	1693
30	Cytopenia	SLE	2051
27	Arthralgia/myalgia	pSS and LLD	1257
33	Proteinuria	SLE and APS	478
18	JIA	JIA/RA*	1528
14	Proteinuria	Lupus nephritis	94

In almost all cases, the pre-test clinical consensus was compatible with the current diagnosis. Remarkably, a lot of different IMIDs have developed in the group of future patients. The mean time between the initial ANA test and the diagnosis is approximately 888 days, which is equivalent to two and a half years.

Higher frequency of positive ANA screening tests in patients with a new diagnosis compared to patients without diagnosis

Using the Chi-square statistic, the proportions of patients with positive ANA screening test results were compared between group A: patients with a new diagnosis, group B: patients with an established diagnosis at baseline or diagnosed around baseline with the ANA test results, and group C: patients with at least one symptom that is relatively common with an IMID at baseline but no current diagnosis (fig. 3).
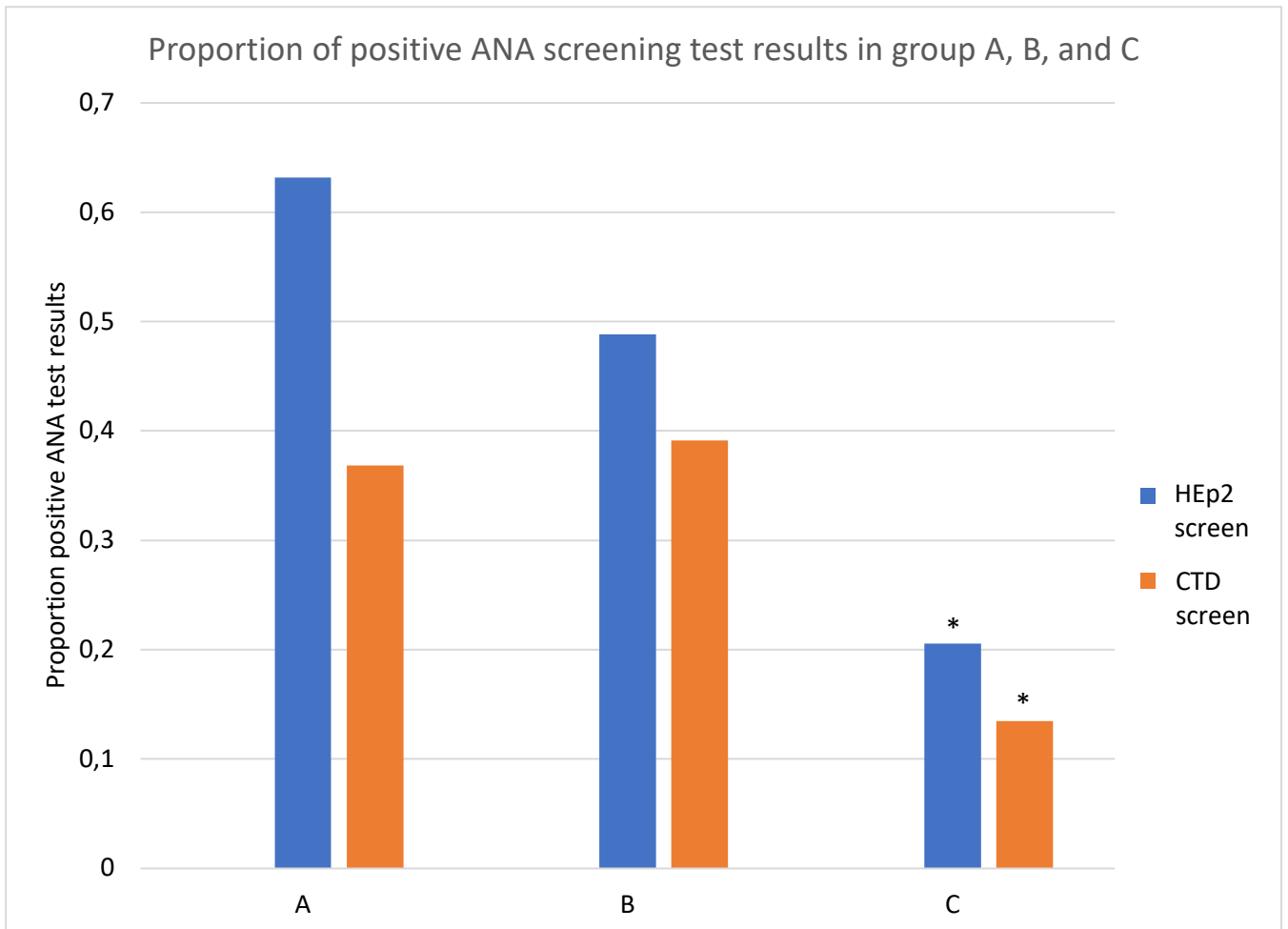

Figure 3: Proportion of positive ANA screening test results in group A, B, and C. Group A: patients with a new diagnosis, group B: patients with an established diagnosis at baseline or diagnosed around baseline with the ANA test results, group C: patients with at least one symptom that is relatively common with an IMID at baseline but no current diagnosis. The Chi-square statistic was used to compare the proportions of patients with positive ANA screening test results between the groups. Significant differences are indicated by *. The blue bars show the proportions of patients with positive HEp2 screen results and the orange bars show the proportions of patients with positive CTD screen results.

For both the HEp2 screen and CTD screen, the proportion of patients with a positive result was larger in the group of patients with a new diagnosis and in the group of patients with an established diagnosis at baseline or diagnosed around baseline with the ANA test results compared to the group of patients without a current diagnosis. Between the group of patients with a new diagnosis and the group of patients with an established diagnosis, the proportions of positive ANA screening tests were not significantly different.

Furthermore, the Chi-square statistic was also used to assess the value of double positive ANA screening test results compared to a single positive ANA screening test result between the different groups (fig. 4). A double positive ANA screening test result is defined as positive test results for both the HEp2 screen and the CTD screen.
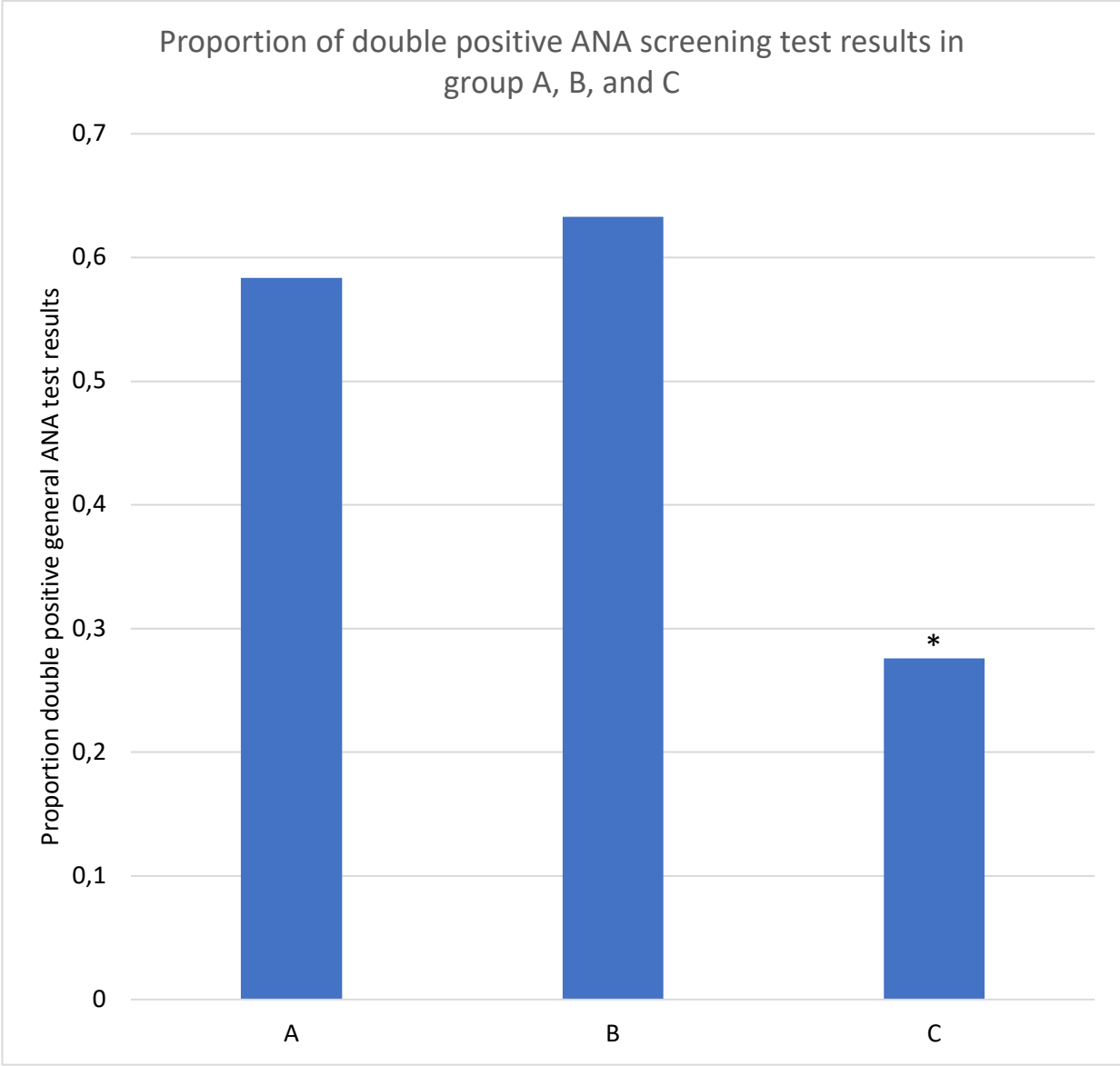

Figure 4: Proportion of double positive ANA screening test results in group A, B, and C. Group A: patients with a new diagnosis, group B: patients with an established diagnosis at baseline or diagnosed around baseline with the ANA test results, group C: patients with at least one symptom that is relatively common with an IMID at baseline but no current diagnosis. The Chi-square statistic was used to compare the proportions of patients with positive ANA screening test results between the groups. Significant differences are indicated by *.

Patients with a new diagnosis and patients with an established diagnosis more often had positive results for both ANA screening tests compared to patients without a diagnosis. Also, the proportion of patients receiving two positive ANA screening test results in the group of patients with a new diagnosis and the group of patients with an established diagnosis did not differ significantly.

The test results for the specific ANA tests were analyzed using the Chi-square statistic (fig. 5). For groups A, B, and C, the specific ANAs compatible with respectively the future-, established-, or suspected diagnosis were determined. Thereafter, the proportion of patients that tested positive for at least one ANA compatible with their future-, established-, or suspected diagnosis were determined. For the patients without diagnosis, compatibility of the ANAs was based on the pretest clinical consensus.
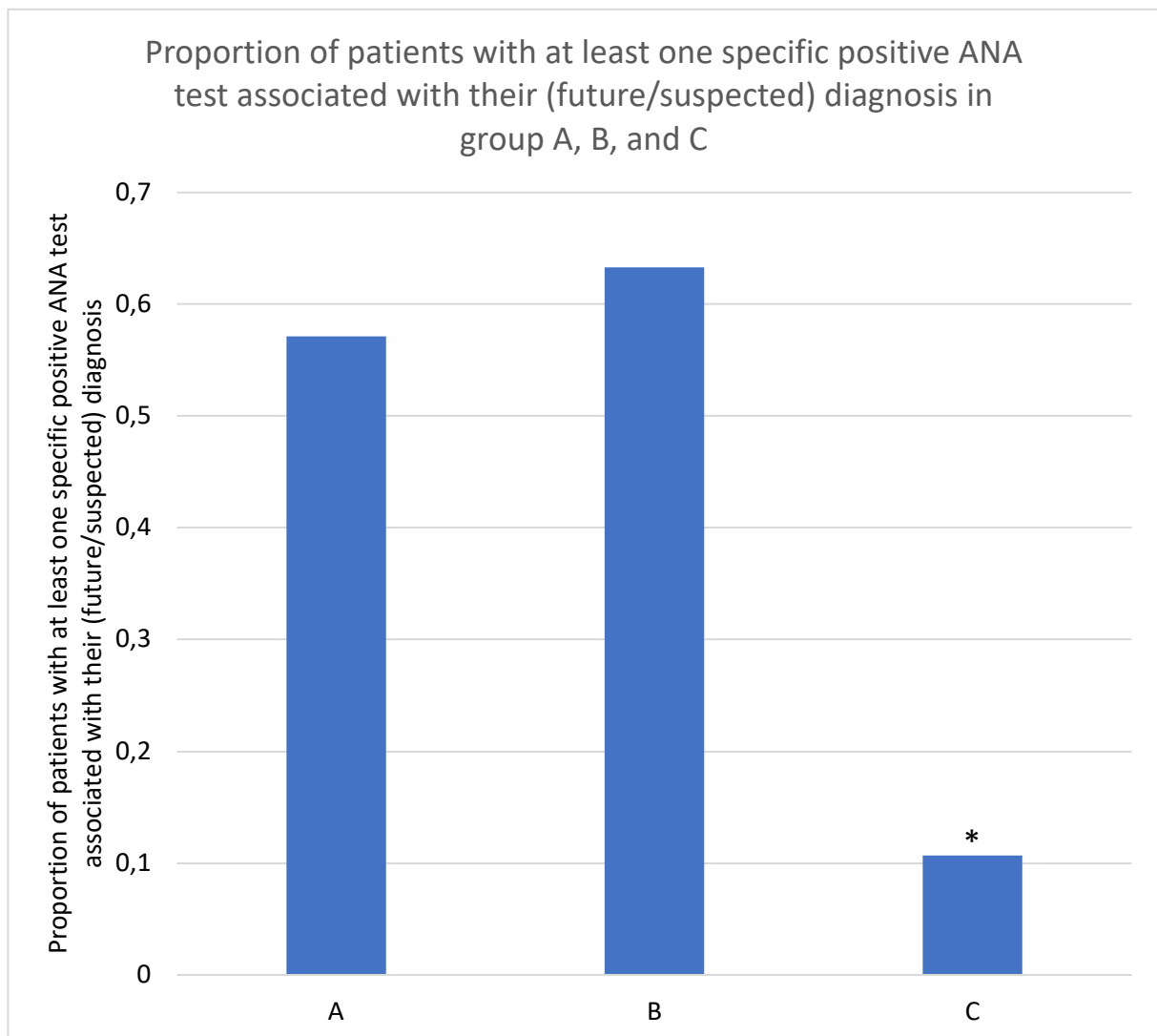

Figure 5: Proportion of patients with at least one specific positive ANA test associated with their (future/suspected) diagnosis in group A, B, and C. Group A: patients with a new diagnosis, group B: patients with an established diagnosis at baseline or diagnosed around baseline with the ANA test results, group C: patients with at least one symptom that is relatively common with an IMID at baseline but no current diagnosis. The Chi-square statistic was used to compare the proportions of patients with at least one positive specific ANA test compatible with their (future/suspected) diagnosis between the groups. Significant differences are indicated by *.

The frequency of positive specific ANA test results compatible with the future-, established-, or suspected diagnosis was significantly higher in the group of patients with a new diagnosis and in the group of patients with an established diagnosis compared to the group of patients without a diagnosis. Furthermore, the proportion of patients with at least one positive specific ANA test compatible with their future- or established diagnosis was not significantly different between the group of patients with a new diagnosis and the group of patients with an established diagnosis.

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For a total of 65 patients, additional ANA test results were obtained. These results were from ANA tests performed in the years after the initial ANA tests were performed. After exclusion of the patients with a suspected-, established-, or future diagnosis with which no ANAs are compatible, 54 patients remained (Table 3).

Table 3: Results from additional specific ANA tests performed in the years after the initial ANA tests were performed. The number of patients with an increase in the number of compatible ANAs, the number of patients without an increase in the number of compatible ANAs, the total number of patients, and the proportion of patients with an increased number of compatible ANAs are displayed. Group A: patients with a new diagnosis, group B: patients with an established diagnosis at baseline or diagnosed around baseline with the ANA test results, group C: patients with at least one symptom that is relatively common with an IMID at baseline but no current diagnosis.

Group	Number of patients with an increase in the number of compatible ANAs	Number of patients without an increase in the number of compatible ANAs	Total number of patients	Proportion of patients with an increased number of compatible ANAs (%)
A	1	6	7	14.3
B	9	21	30	30.0
C	3	14	17	17.6

An important consideration is that in contrast to the baseline specific ANA tests, only a lineblot was conducted in the subsequent ANA tests that were performed. This is because the lineblot is the standard procedure used at the University Medical Center Utrecht. Consequently, new ANAs that can solely be detected using the CTD single analyte could not be detected in subsequent specific ANA tests. In addition, the lineblot or CTD single analyte was not performed in all patients at baseline, because the corresponding ANA screening test result was negative. It was therefore assumed no ANAs were present.

Using the Chi-square statistic, the proportions of patients with an increased number of compatible ANAs were compared between the groups. It was established these proportions were not significantly different, indicating that an increase in the number of compatible ANAs is not a predictor of future IMID development. As this statistical analysis was performed on a small group, the conclusions of these data remain inconclusive. It was therefore decided to discuss some of the interesting cases from group A (Table S1) individually (Table 4).

Table 4: Summary of the results obtained from three patients in group A. The pre-test clinical consensus, current diagnosis, HEp2 screen results, the time between the initial and subsequent screening tests, initial CTD screen result, specific ANA test results, the time between the initial and subsequent specific ANA tests, and the time between the ANA tests and the diagnosis are displayed for three cases from group A. Important to note is that in subsequent specific ANA tests only lineblots were performed, because this is the standard procedure. 'X' indicates the test was not conducted. 'neg for all' indicates that no ANAs were detected when a specific ANA test was performed. For the ANA screening tests, green indicates the test was positive, red indicates the test was negative, and orange indicates the test was weakly positive.

	Pre-test consensus	Current diagnosis	HEp2 screen result at baseline	CTD screen result at baseline	Subsequent HEp2 screen result	Time between initial and subsequent HEp2 screen (days)	Specific ANAs at baseline	Specific ANAs detected subsequently	Time between initial and subsequent specific ANA tests (days)	Time between ANA test and diagnosis (days)
1	Cytopenia	SLE			X	X	Single analyte: U1-RNP; RNP70 Lineblot: U1-nRNP/Sm (strong); Ribosomal-P (weak); SSA-60 (weak)	SSA-60 (weak); U1-nRNP/Sm (strong)	1994	1980
2	Proteinuria	SLE and APS				500	Single analyte: PCNA Lineblot: PCNA	dsDNA (weak) PCNA	500	478
								PCNA (strong)	1144	
3	Proteinuria	Lupus nephritis				70	Single analyte: X Lineblot: PM-Scl (weak); SSA-52 (weak)	PM-Scl (weak); SSA-52 (weak)	70	94
						147		X	X	
						324		X	X	
						422		X	X	
						653		X	X	
						777		X	X	
						868		X	X	
						1053		X	X	
						1158		PM-Scl (weak)	1158	
						1235		PM-Scl (weak)	1235	
						1375		X	X	
						1508		PM-Scl (weak)	1508	
						1596		X	X	

Case 1

Case 1 concerns a patient that was diagnosed with SLE. Over five years prior to diagnosis, unti-U1-RNP and anti-RNP-70 antibodies were detected using the CTD single analyte method. With the lineblot, anti-U1-nRNP/Sm antibodies, anti-ribosomal-P antibodies, and anti-SSA-60 antibodies were detected. Approximately five and a half years after the initial ANA tests were conducted, a lineblot was performed. This test revealed the presence of anti-SSA-60 antibodies and anti-nRNP/Sm antibodies. It should be noted anti-ribosomal-P antibodies were not detected in the second lineblot even though they were detected initially. Important to note is that the absence of anti-U1-RNP antibodies and anti-RNP70 antibodies cannot be confirmed by these results, as only a lineblot was performed, which does not contain the antigens required for detection of these antibodies.

Based on literature, it is expected that the number of ANAs compatible with the future diagnosis increases in the time leading up to the diagnosis. For this case, this pattern does not seem to apply. Contrasting with the expectations, the anti-ribosomal-P antibodies seem to have disappeared. However, close examination of this case reveals that the results are not as surprising as they appear. In the study conducted by Arbuckle and colleagues[11] it was demonstrated that the anti-Ro antibodies, such as anti-SSA-60 antibodies, are present in approximately 70% of the patients five years prior to diagnosis. It is therefore no surprise these ANAs are detected in this case five years prior to diagnosis. However, it is remarkable anti-U1-nRNP/Sm antibodies are detected in this patient five years prior to diagnosis, as these ANAs are likely to appear months before diagnosis. The other ANAs taken into consideration by the study all appear in a large proportion of the patient years before clinical diagnosis. When these ANAs are not present five years before clinical diagnosis, these ANAs are not very likely to appear in the years leading up to the diagnosis. It is therefore not very surprising that in this case no additional ANAs were found around the time of diagnosis compared to the ANAs found five years prior to diagnosis. Hence, the results from the current study do not rule out the results from the previously performed study and vice versa.

Case 2

Case 2 concerns the only patient of which is known that an additional ANA compatible with the diagnosis was gained around the time of diagnosis. Approximately one and a half years before clinical diagnosis of SLE and APS, anti-PCNA antibodies were detected in the patient. Around the time of diagnosis, the patient was tested positive for anti-dsDNA antibodies and anti-PCNA antibodies. Approximately three years after the initial ANA tests were conducted, only anti-PCNA antibodies were detected. This case illustrates ANAs may appear and disappear, once more emphasizing the plasticity of ANAs and complexity of autoimmune diseases such as SLE.

Case 3

This case is of particular interest, because ANA tests were performed regularly over the course of almost four and a half years. The patient initially got negative ANA screening test results at baseline. The lineblot performed at baseline demonstrated the presence of anti-PM-Scl antibodies and anti-SSA-52 antibodies. Only two months after these initial tests, the second lineblot was conducted, yielding the same results. Shortly after, the patient was diagnosed with lupus nephritis, which is a condition in context of SLE. In the four years after clinical diagnosis, HEp2 screening tests were performed an additional twelve times to confirm the presence of ANAs. This is of interest, as patients diagnosed with SLE are likely to have ANAs associated with SLE in the years prior to diagnosis. However, after clinical diagnosis, patients are not very likely to gain additional associated ANAs. More importantly, finding new ANAs would be clinically irrelevant unless the clinical condition of the patient has changed. In light of the current diagnostic strategy of IMIDs, this patient is an example of a case in which ANA tests are performed without diagnostic relevance. Exceptions to this could include suspicion of an overlap syndrome or changes in the clinical manifestations. This seems unlikely for this case, as the screening tests were performed every few months.

Conclusions and discussion

The aim of the current study was to evaluate the predictive value of positive ANA screening tests and specific ANA tests for IMID development over the course of six years. In order to achieve this aim, the cohort of the study conducted by Otten and colleagues[8] was reevaluated using additional information retrieved from the electronic health records on current diagnoses of these patients. It was found that for 19 patients out of the cohort consisting of 1030 patients, a new diagnosis was established in the six years after the initial ANA tests were performed.

The results from the ANA screening tests and specific ANA tests were considered. It was found that patients with a new diagnosis and patients with an established diagnosis at baseline more often had (double) positive ANA screening test results and positive specific ANA tests results compared to patients without a diagnosis. This is an interesting observation, as patients with a new diagnosis and patients without a diagnosis originally belonged to the same category, according to the study conducted by Otten and colleagues[8]. Hence, it appears patients that will develop an IMID in the future are more likely to get positive ANA screening test results and positive specific ANA test results for ANAs compatible with their future diagnosis in the years preceding clinical onset of the IMID. Besides, these results reveal that patients that will be diagnosed with an IMID in the future are similarly likely to have positive ANA test results compared to patients with an established diagnosis. This observation illustrates that the ANA profile is already present in the years preceding clinical diagnosis. Positive ANA screening tests and specific ANA tests could therefore be of potential use in early diagnostics of individuals with a high pretest probability of developing an IMID in the future.

The data also demonstrated that the proportion of patients with an increase in the number of compatible ANAs is not different between patients with a new diagnosis, patients with an established diagnosis, and patients without a diagnosis, which suggests that an increase in the number of compatible ANAs is not a predictor of future IMID development. It should be noted this statistical analysis was performed on a group consisting of 54 individuals. Due to this small sample size, the reliability of the statistical analysis is limited in addressing whether an increase in the number of compatible ANAs could be a predictor of future IMID development. In order to approach this matter properly, a larger sample size is highly recommended. Besides, patients with a new diagnosis were diagnosed within six years after the initial ANA tests were performed. Based on previous literature, ANAs could already be present up to ten years before clinical onset of IMIDs[10]. Hence, it is likely that six years prior to clinical diagnosis the ANAs were already present. This could also be one of the reasons new compatible ANAs rarely appeared in the years leading up to clinical diagnosis.

It should be noted that ANAs are present in 5-10% of the healthy individuals[13] and that different ANAs may be associated with various IMIDs. For these reasons, positive ANA test results cannot be used as the only predictors of future IMID development. Clinical guidance based on the presentation of the patient remains the most important aspect in establishing a diagnosis. However, the data from this study indicate positive ANA test results can play a role in confirming the clinical suspicion. For instance, patients with relatively mild symptoms that are associated with an IMID, positive ANA screening tests, and positive results for specific ANAs compatible with the same IMDI could provide reasons to monitor patients for the development of the suspected IMID. Hence, positive ANA test results can be used to identify patients that are at risk of developing an IMID at an early stage.

A few limitations to this research are important to consider. The most important one to be the small group of patients that developed a new diagnosis. From a total of 1030 patients that were included in the original cohort, only 19 patients developed a new diagnosis. Due to the small sample size, conclusions based on statistical analyses remain dubious. In addition, these patients developed a large variety of different IMIDs, posing a challenge on drawing conclusions that apply to all these patients. A text mining algorithm was used to analyze the electronic health records of patients included in the study to determine the current diagnoses of the patients. Even though the text mining tool has a high success rate, it is not perfect, resulting in possible losses of diagnoses. In addition, some patients that were included in the original cohort left the hospital, resulting in a loss to follow up. For these cases it remains unknown whether the patients have developed an IMID. This results in an

underestimation of the number of patients with a new diagnosis. Another limitation to this research concerns the information that was retrieved from the patients in the years after the initial ANA tests. In subsequent ANA tests, only the HEp2 screening and lineblot were performed. Consequently, ANAs that can solely be detected using the CTD single analyte cannot be detected in the subsequent specific ANA tests, hence causing an underestimation of patients in which the number of compatible ANAs has increased. Besides, a lineblot or CTD single analyte were only performed at baseline when the corresponding ANA screening test result was positive. This could have caused an overestimation of the increase in the number of compatible ANAs over the years.

Even though the group of patients that developed a new diagnosis was small to conduct proper statistical analyses, it is quite a large group compared to the total number of patients included in the original study. This is due to the methods of selection used to include the patients of this study. For studies that aim to investigate the course of development of IMIDs, this method could potentially be used to identify patients that are at high risk of developing an IMID in the near future. Another strength to this research is the large follow-up time of six years. On average, patients developed an IMID two and a half years after the initial ANA tests were conducted. This provides a relatively large window to predict future IMID development based on ANA test results conducted two and a half prior.

The results from the current study have demonstrated ANAs are indeed more often present in future IMID patients compared to patients that will not develop an IMID. A combination of a positive ANA test result and a high pretest probability for an IMID based on clinical manifestation may therefore provide an indication that patients should be monitored for the development of an IMID in the future. As the ANAs were already present at baseline, this study design does not allow for investigation of the immunopathology leading up to the clinical diagnosis. In order to find out more about the immunological changes that occur in the years prior to clinical diagnosis, patients that are at high risk for IMID development should be identified at an earlier stage. Currently, no strategy is available to identify these patients, because factors that put individuals at risk remain unknown. For future research, it is therefore of high interest to identify the risk factors associated with IMID development. Once these patients can be identified, they can be monitored over time, providing an opportunity to closely examine IMID development. This will pave the way towards unraveling the complex disease mechanisms driving IMIDs.

Future perspectives

Some interesting points of discussion have come along in this study that provide opportunities for future research. First of all, when the additionally retrieved data were analyzed it became apparent that specific ANA tests were often repeated at small intervals, which did not result in the detection of additional ANAs. For instance, 30 patients with an established diagnosis with which ANAs are associated were repeatedly tested without relevant outcomes. A total of 6 patients were tested 4-8 times of the course of 6 years and 4 patients were tested 13-19 times over the course of 6 years. These tests did not yield any clinically relevant results. Besides, in patients with an established diagnosis, it is not expected many new ANAs are discovered. More importantly, without a change in the clinical manifestation or suspicion of an overlap syndrome, newly appearing ANAs are clinically irrelevant. In some of these cases, patients were tested every few months, which makes it unlikely patients had changes in their clinical condition on every occasion. Hence, ANA tests seem to have been conducted repeatedly without clinical benefit.

In a study conducted in 2020, the utility of repeated ANA tests was explored. The authors evaluated a total of 370,000 ANA tests, 21% of which were repeats. In only 10% of these repeats changed the result from negative to positive, indicating that negative ANA test results are likely to remain negative upon repeat testing. Only 1.1% of the repeat tests proved to be clinically relevant. Based on their results, the authors concluded that repeat testing had a low clinical positive predictive value for the diagnosis of a new ANA associated rheumatological disorder[14]. In combination with the results from the current study it can be established that the utility of repeat testing is an area of interest for future research in order to improve IMID diagnostics.

Another point of interest is the use of ANA tests in patients with an established diagnosis with which no ANAs are associated. During the data analysis, it became apparent that half of the patients with an established IMID were diagnosed with an IMID with which no ANAs are associated. The vast majority of these patients were diagnosed with either RA or JIA. As no ANAs are associated with these conditions and it is known that ANAs appear in healthy individuals as well, it is interesting ANA tests were requested for these individuals in the first place. A possible reason to request ANA tests could be suspicion of an overlap condition. As these conditions are rare, it seems unlikely this was the case for half of the patients with an established diagnosis. Other than the lack of utility of performing these tests, unexpected positive results can also cause confusion regarding interpretation. This will ultimately hamper the diagnostic process and possibly also the treatment strategy.

Based on these two observations, it is proposed that future research may focus on the development of guideline that can be implemented in the clinic. These can provide guidance on when to request ANA tests, hence ensuring ANA tests are performed when relevant. This will ultimately improve IMID diagnostics.

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Table S1: Summary of the results obtained from patients in group A. The pre-test clinical consensus, current diagnosis, HEp2 screen results, the time between these tests, initial CTD screen result, specific ANA test results, the time between these tests, and the time between the ANA tests and the diagnosis are displayed. Important to note is that in subsequent specific ANA tests only lineblots were performed, because this is the standard procedure. 'X' indicates the test was not conducted. 'neg for all' indicates that no ANAs were detected when a specific ANA test was performed. For the ANA screening tests, green indicates the test was positive, red indicates the test was negative, and orange indicates the test was weakly positive.

Pre-test clinical consensus	Current diagnosis	HEp2 screen result at baseline	CTD screen result at baseline	Subsequent HEp2 screen result	Time between initial and subsequent HEp2 screen (days)	Specific ANAs at baseline	Specific ANAs detected subsequently	Time between initial and subsequent specific ANA tests (days)	Time between ANA test and diagnosis (days)
Serositis	MCTD				714	Single analyte: dsDNA; SSA-60 Lineblot: SSA-60 (weak); U1-nRNP/Sm (strong)	U1-nRNP/Sm (strong)	1418	128
Raynaud	SSc				1049	Single analyte: X Line blot: neg for all	X	X	322
PMDM	PMDM and SSc				616	Single analyte: neg for all Lineblot: PM-Scl	Neg for all	903	902
Liver function disorder	RA			X	X	Neg for all	X	X	230
APS	SLE				1838	X	X	1838	1776
Sicca	pSS/SSc			X	X	Single analyte: CENP-B Lineblot: CENP-B (strong)	X	X	604
cytopenia	SLE			X	X	Single analyte: U1-RNP; RNP70 Lineblot: U1-nRNP/Sm (strong); Ribosomal-P (weak); SSA-60 (weak)	SSA-60 (weak); U1-nRNP/Sm (strong)	1994	1980
Arthralgia/myalgia	RA			X	X	Neg for all	Neg for all	917	610
Skin abnormalities compatible with SLE	PM and CLE				564	X	X	X	52

Sicca	SLE	X			X	Single analyte: X Lineblot: PCNA	PCNA	1272	362
APS	SLE and APS				2008	Single analyte: dsDNA Lineblot: dsDNA (weak); nucleosomes (weak)	Nucleosomes (weak)	2008	1127
Arthralgia/myalgia	PMR	X			X	Single analyte: X Lineblot: neg for all	X	X	289
Sicca	pSS	X			X	Neg for all		1330	1371
Interstitial lung disease	RA	X			1469	X	Neg for all	1469	1693
Cytopenia	SLE				1973	Single analyte: X Lineblot: SSA-52 (weak)	SSA-52	1973	2051
Arthralgia/myalgia	pSS and LLD				1809	Single analyte: dsDNA; Mi-2 Lineblot: SSA-52 (weak)	Neg for all	1809	1257
Proteinuria	SLE and APS				500	Single analyte: PCNA Lineblot: PCNA	dsDNA (weak) PCNA	500	478
							PCNA (strong)	1144	
JIA	JIA/RA	X			X	X	X	X	1528

	70	147	324	422	653	777	868	1053	1158	1235	1375	1508	1596
PM-Scl (weak); SSA-52 (weak)	70	X	X	X	X	X	X	X	1158	1235	X	1508	X
Single analyte: X Lineblot: PM-Scl (weak); SSA-52 (weak)		X	X	X	X	X	X	X	PM-Scl (weak)	PM-Scl (weak)	X	PM-Scl (weak)	X
Lupus nephritis													
Proteinuria													