



ANA and Antibody Series

The Puzzle of ANA-Negative Systemic Scleroderma

Author's Note

I decided to write this article when I first read the new Salazar et al. (2015) paper “Antinuclear antibody-negative systemic sclerosis” before it was formally published. I was very happy to see a large scale study which focused on the relatively rare situation where patients clearly exhibit symptoms consistent with the diagnosis of systemic scleroderma, but repeatedly test negative for antinuclear antibodies. However, once I read the article in detail I realized that while there is important information to be learned from this study, there is also a major problem with the study from a research perspective and decided to use this as an opportunity to educate people about common research problems in addition to highlighting the main topic: antinuclear antibody (ANA)-negative systemic scleroderma.

Background

Depending on the research study, between 5% and 10% of patients with typical scleroderma symptoms repeatedly test negative for ANA when done by the best testing method currently available – indirect immunofluorescence (IFA). This paper explores the nature and frequency of ANA-negative scleroderma and also speculates on what might explain these rare cases.

Negative ANA versus “Negative” ANA

Before we look at the research on patients that repeatedly test negative for ANA, we first need to clarify what we mean by negative ANA. This topic is covered in great detail in some of the other papers in my *ANA and Antibody Series*, but here is a brief overview of the problem:

- ANA testing is currently done by three different methods: indirect immunofluorescence (IFA), ELISA, and Multiplex.
- ANA testing by IFA has been done for decades and can simultaneously detect the presence of 100 to 150 different anti-nuclear antibodies (Shanmugam et al 2011), but can't specifically identify exactly what antibody (or antibodies) have been detected. The result of an ANA test done by IFA is either negative or positive, reported as a titer (dilution factor) such as 1:320. A positive ANA by IFA includes both the titer and a “staining pattern”. This staining pattern can suggest the type of antibody present and potentially the general type of autoimmune disease, but interpreting staining patterns is a bit of an art form and is not always exact. Additional testing is usually needed to determine the specific antibody/antibodies that have been detected by a positive IFA ANA test. ANA by IFA is time consuming, needs well trained technicians, and is (therefore) expensive.
- Historically, ANA testing was originally discovered and performed using IFA. Now, however, most ANA testing is (by default) done using newer, less expensive and less

"hands on" methods such as ELISA or Multiplex. In contrast with ANA by IFA's ability to detect the presence of more than 100 antibodies (but not identify specific ones), ANA testing by ELISA detects and identifies 8 to 10 specific antibodies while testing by Multiplex detects and identifies 11 to 13 specific antibodies.

- ANA testing by ELISA or Multiplex is very accurate IF the patient has one of the antibodies included in their respective panels - or the ANA will be reported as negative, thus suggesting that the patient does not have an autoimmune disease. This is especially true for scleroderma patients as there are now at least 8 different antibody variants of scleroderma that can only be detected when ANA testing is done by IFA. General ANA screening tests usually include at most two scleroderma-specific antibodies (Scl-70 and centromere) thus potentially missing identifying a significant number of patients with other scleroderma antibody variants that have a different symptom profile and prognosis.
- For purposes of this paper, when we write about ANA-negative scleroderma, we are *only* considering cases where the testing is done by IFA, the most reliable method for detecting ANA and the method recommended by the American College of Rheumatology as the "gold standard" for ANA testing.

ANA-Negative Scleroderma – The Research

Historically, a number of research studies have consistently suggested that between 5% and 10% of patients, who clearly meet the criterion for the diagnosis of systemic scleroderma, test negative for ANA when done by IFA. Most of these studies have included relatively small groups of patients. However, a just published study (Salazar et al. 2015) looked at the demographic and clinical characteristics of 3249 patients enrolled in the Scleroderma Family Registry. This study included patients mostly from the US and about 10% from Canada. Of the 3249 patients, 208 (6.4%) of the patients were ANA-negative, consistent with previous studies.

The significant* (see below for a discussion on "significance" in research studies) findings of this study include:

- Patients in the ANA-negative group were likely to be male (p<.008).
- There was more "generalized" diffuse skin involvement in the ANA-negative group.
- ANA-negative patients had more malabsorption issues.
- ANA-negative patients had lower rates of telangiectasias, digital ulcers, and vasculopathy.
- Interestingly, there was no significant difference in scleroderma renal crisis, pulmonary fibrosis, or overall survival rates.

Digression – Let's Talk About Research Design

Here is the actual abstract of the Salazar study:

Objective: To examine the demographic and clinical characteristics of systemic sclerosis (SSc) patients without antinuclear antibodies (ANA) compared to ANA-positive patients.

Methods: SSc patients enrolled in the Scleroderma Family Registry and DNA Repository were included. Relevant demographic and clinical data were entered by

participating sites or obtained by chart review. ANA and SSc-related antibodies were determined in all investigated patients using commercially available kits at our laboratories.

Results: This study included 3249 patients, of whom 208 (6.4%) were ANA negative. The proportion of male patients was higher in the ANA-negative group (OR = 1.65; $p = 0.008$). ANA-negative patients experienced less vasculopathic manifestations of SSc. The percent predicted diffusing capacity of carbon monoxide (DLCO) was higher in ANA-negative patients ($p = 0.03$). Pulmonary arterial hypertension (PAH) per right heart catheterization was less common in the ANA-negative group (OR = 0.28; $p = 0.03$). Furthermore, patients with negative ANA had a lower prevalence of telangiectasias and digital ulcers/pits (OR = 0.59, $p = 0.03$ and OR = 0.38, $p = 0.01$, respectively). Although diffuse cutaneous involvement was more common, the modified Rodnan Skin Score (mRSS) was lower in the ANA-negative group (2.4 points lower, $p = 0.05$). Furthermore, they experienced more malabsorption ($p = 0.05$). There was no difference in the frequency of pulmonary fibrosis or scleroderma renal crisis. All-cause mortality was not different between the 2 groups ($p = 0.28$).

Conclusions: In conclusion, the results of this study suggest that SSc patients who are ANA negative constitute a distinct subset of SSc with less vasculopathy (less PAH, digital ulcers, and fewer telangiectasias), a greater proportion of males, and possibly, more frequent lower gastrointestinal involvement.

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Depending on how you define systemic scleroderma, which does vary among different researchers, there are as many as eight different variants of systemic scleroderma based on antibody type. Some of the most common antibodies, representing the vast majority of all diagnosed cases of systemic scleroderma, include Scl70 (topoisomerase), centromere, RNA Polymerase III, TH/To, and U1-RNP. While there is definitely some clinical similarity/overlap between all of these variants of scleroderma, there are also vast differences in many clinical characteristics ranging from degree of skin involvement, likelihood of scleroderma renal crisis, and longevity.

Because of the heterogeneous nature of the various antibody-specific scleroderma variants, *it makes no sense from a research design perspective to compare a measure such as mortality rates for the ANA-negative systemic scleroderma group with mortality rates of the combined ANA-positive group since mortality rates vary widely among the antibody-specific scleroderma variants.* As it turned out, there was no significant difference in mortality rates between the ANA-positive and the ANA-negative groups, but this is not really useful information from either a clinical or research perspective because of the great heterogeneity of mortality rates among the different ANA-positive scleroderma variants.

In contrast to the Salazar study, Hamaguchi et al. (2008) looked at the clinical symptoms associated with six antibody-specific scleroderma variants plus an ANA-negative group. This makes more sense from a research and clinical perspective because it allows researchers to better understand the potential different clinical manifestations of each scleroderma variant, including ANA-negative patients.

There is actually a second problem with the Salazar study as well as other studies that have looked at the characteristics of ANA-negative systemic scleroderma patients that is potentially even more significant than failing to compare the specific scleroderma antibody-defined subgroups. In all of these studies, an implicit assumption is made that the group of patients who are ANA-negative are a homogeneous group. While that is one possibility, it is at least as likely that the ANA-negative group consists of unique variants of systemic

scleroderma which may, like the eight identified ANA-positive variants, have distinct and potentially different clinical profiles. Since no data is presented to support the assumption that the ANA-negative group is a homogeneous group, interpretation of studies such as the Hamaguchi study that compared the ANA-negative group with individual antibody specific scleroderma variants has to be considered in light of the possibility that the ANA-negative may ultimately be determined to be heterogeneous with yet undiscovered antibodies.

Back to the Topic of ANA-Negative Systemic Scleroderma

As noted earlier in this paper, ANA testing by IFA is currently almost always done using the HEp-2 substrate that detects the presence of between 100 and 150 different antibodies. The Salazar paper notes that “It is also possible that ANA-negative patients have other antibodies that are not currently detected by our traditional assays...”. It seems reasonable that this explanation may well be correct, raising the possibility that future improvements in ANA testing methodology could lead to the discovery of additional antibodies that will eventually be associated with new variants of systemic scleroderma but are not currently detectable by IFA.

One other potential explanation for some of these IFA ANA-negative patients is that some may have an extremely rare Primary Immunodeficiency Diseases (PID), e.g., Common Variable Immunodeficiency (CVID). With this syndrome, a patient has significantly reduced lymphocytes or plasma cells that produce antibodies and, as a result, often tests negative on ANA. Recent research (Boyle et al. 2007) indicates a prevalence in the US of PID of about 1 in 1,200. With CVID, about 15-20% of these patients may develop some form of autoimmune disease, including scleroderma (Lewandowicz-Uszynska et al. 2007). While it is unlikely that PID accounts for a large number of ANA-negative scleroderma cases, it should be considered a possibility for at least some of these rare cases.

In spite of the above, I can't really fault the researchers for including statistically accurate but non-useful comparison data in this study. In getting papers published in a major research journal, it is very unlikely that an article which noted only one major significant finding (6.4% of scleroderma patients were ANA-negative by IFA) would be accepted for publication. While I contend that there is minimal research value in comparing average clinical attributes of a heterogeneous ANA-positive scleroderma patients against ANA-negative patients, if it does ultimately turn out that the ANA-negative patient group is a homogeneous population, then finding that ANA-negative patients tended to have less vasculopathy (blood vessel damage) and be on the “milder” end of the spectrum will be important information from both a research and clinical perspective. If, however, the ANA-negative group contains two or more distinct (currently undetectable) antibody types, then the data will be even less useful unless future improved ANA testing methods are able to detect and identify these new distinct scleroderma antibody subtypes.

Conclusion

Bottom line - there is one key and relevant piece of information that can be gleaned from the Salazar et al. study: in a large group of systemic scleroderma patients, only 6.4% of the patients test negative for ANA by IFA, thus replicating the findings of several previous smaller studies. This is very important information that may help more patients get correctly diagnosed with “atypical/ANA-negative” scleroderma. Many rheumatologists firmly believe that you have to have a positive ANA to securely diagnose scleroderma. While this is usually true, given the fact that about 5% of scleroderma patients are ANA-negative, clinicians should consider the diagnosis of “atypical/ANA-negative” scleroderma when faced with patients who have symptoms that strongly suggest systemic scleroderma but repeatedly test ANA-negative.

***A Note About “Significant” Results in Research Studies**

We often see headlines in the popular press or online with titles like “New Drug Significantly Improves Lifespan of Late Stage Colon Cancer Patients!”. If you read the original article you may find that the article abstract really says something like “in a pool of 87 stage V colon cancer patients, xiziphinate hexachloride increased average survival time from 4 months to 5 months”.

The article headline is correct, but misleading. In the world of research, the word “significant” is a statistical term with a precise meaning. Typically, it means that there was only a 5% chance that the results of the study were *not* due to chance. To make this a bit clearer, if this cancer study was done with a group of only 10 patients, then it is much more likely that the one-month extension of survival time is just from chance, and if you picked another random group of 10 patients, then you might not see the same results. However, if the study was done with 1000 patients, then it is extremely unlikely that this one-month increase in survival time was just from the chance makeup of the subject group, and was “statistically significant”.

Basically, the word “significant” has nothing to do with clinical significance, but rather statistical significance. Is the one-month extension of lifespan “significant” to a stage V colon cancer patient? It may well be, but that is a judgment on the part of the patient and has little to do with the use of the scientific term “statistically significant”.

To illustrate, if we look within the field of scleroderma research, a well-designed research study on the effects of cyclophosphamide (Cytoxan) on lung function and other disease-related symptoms (Tashkin, et al. 2006) noted significant improvement in two important measures of lung functioning vs. the placebo group at 12 months; a third measure did not reach statistical significance. On one of the measures (total lung capacity) in the Cytoxan group, the value stayed the same as baseline at 12 months - around 70% of normal. By contrast, the placebo group dropped from 68% to 65%, statistically significant but not clinically significant to the patient.

Also, the researchers didn’t consider the potential long-term harm to the patient from Cytoxan suppressing their immune system. While the article abstract talked about the “significant but modest beneficial effect on lung function...” and mentioned that the effects “were maintained through the 24 months of the study”, it was only in the last sentence of the article itself that the authors noted that “Caution regarding the use of cyclophosphamide is still warranted, since potential long-term consequences were not evaluated.” Patients need to be aware of this problem when they are working with their physicians in devising treatment plans. It is always important to understand the potential for long-term treatment complications, especially when a particular treatment might provide negligible real-world benefits.

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