ANCA as a predictor of a relapse: useful in patients with renal involvement but not in patients with non-renal disease. - Supplementary data

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Methods

Inclusion criteria for patients

All patients who visited the clinic at the Maastricht University Medical Center (MUMC) between January 1, 2000 and November 1, 2011 were evaluated. Inclusion criteria were a diagnosis for primary ANCA associated vasculitis (AAV) according to the revised Chapel Hill Consensus Conference nomenclature and a positive PR3- or MPO-ANCA according to the algorithm described before.[1,2] Exclusion criteria were incomplete follow-up data and/or refractory disease.[3]

Classification of patients

Patients were subclassified using the EMEA classification into Granulomatosis with Polyangiitis (GPA; Wegener's), Microscopic Polyangiitis (MPA) or Eosinophilic Granulomatosis with Polyangiitis (EGPA; Churg Strauss Syndrome).[4] Renal involvement was preferably determined by a kidney biopsy showing pauci-immune necrotizing glomerulonephritis.[5] However, surrogate markers such as haematuria in combination with red cell casts, dysmorphic erythrocytes (>10) and/or proteinuria sufficed.[4] All patients have been treated according to the European League Against Rheumatism (EULAR) guidelines.[6] With regards to the induction therapy, we grouped cyclophosphamide (oral or IV), mycophenolate mofetil and rituximab as "aggressive" and all other therapies as "mild" (.e.g. methotrexate). Maintenance therapy was in most patients continued for 18 months and consisted in most patients of azathioprine (but occasionally mofetil mycophenolate or methothrexate was given when azathioprine intolerance was present). Steroids were tapered down to at least 7.5mg according to protocol[7] and thereafter either stopped or tailored.[8] Patients were defined as persistently ANCA positive (PP) when ANCA values remained positive during follow-up and as non-persistently ANCA positive (NPP) when an ANCA measurement had at least once been negative. Sampling interval was categorized in either ≥4 measurements per year or less.

Disease activity state

The definitions recommended by the EULAR of 2007 were applied to further define disease activity states.[3] Disease activity was scored using the BVAS v3.[9] Remission was defined as absence of disease activity attributable to active disease during maintenance immunosuppressive therapy of a prednisone dosage of 7.5mg or lower. A relapse was defined as re-occurrence or new onset of disease attributable to active disease combined with an increase or addition of immunosuppressive treatment. Relapses were further subdivided in minor or major, depending on whether the relapse

was potentially organ- or life threatening or not.[3] For a renal relapse, the (re)occurrence of haematuria in combination with a rise in serum creatinine of 25% was required to be characterized as a major relapse.[10]

ANCA measurements

Patients were routinely evaluated during follow-up, generally every three months during the first two years after diagnosis and/or a relapse and 2-3 per year later on. At every visit, patients were screened for potential symptoms of a relapse[3] and blood was drawn.

Several tests to detect the presence of ANCAs were performed throughout the study.[1] ANCA were detected by indirect immunofluorescence (IIF) on ethanol-fixed neutrophil granulocytes (INOVA Diagnostics, San Diego, CA).[1] Samples were diluted serially in PBS, starting at 1:16. Antigen-specific solid-phase ANCA tests were performed for the detection and quantification of anti-PR3 and anti-MPO ANCA. Initially, commercially available direct PR3- and MPO-ANCA enzyme linked immunosorbent assays (ELISA) were used (Euro-diagnostica, Malmö, Sweden).[11] On 1-10-2005, this assay was replaced by a fluorescent-enzyme immune-assay (FEIA) for PR3- and MPO-ANCA (EliA, Thermo Fisher, Freiburg, Germany).[1] During the transfer, ANCA measurements were performed using both methods. For quantitation of MPO- and PR3-ANCA the samples were diluted 1:50 in PBS as instructed by the manufacturer. Results were calculated in arbitrary units by a standard curve. Patient samples with results above the highest standard were further tested in two-fold dilutions as appropriate. Intra- and inter-assay variation is shown in table S1.

Definition of an ANCA rise

For the detection of an ANCA rise, the value was compared to all measurements made with the same assay in the past 6 months. Next to ANCA rises as detected by antigen-specific assays, we additionally investigated ANCA rise as using the IIF technique. For the IIF, a rise was defined as a fourfold increase.[12] For the antigen-specific solid-phase assays, we defined a rise using the slope of an increase **(figure S1)**, thereby taking into account the relative increase (in %) and the time between measurements (in days). A receiver operating characteristics (ROC) curve was calculated to determine the optimal cut-off value of the slope. To ensure that small elevations were above the intra-assay coefficient of variation, a rise had to constitute to a relative increase of at least 25% and an absolute increase equivalent to a doubling of the lowest value of a borderline result (at least 10 AU for the ELISA and 5 U/ml for the FEIA). Because our analysis is focused on patients in

remission, only serum samples drawn at least 3 months after the previous disease activity were eligible for detection of an ANCA rise.

A receiver operating characteristics (ROC) curve was calculated to determine the optimal cut-off value of an ANCA rise. To do so, we had to transform the longitudinal follow-up in dichotomous periods of remission and relapse (state variable). As explained in **figure S2**, we did so by splitting the follow-up in different timeframes of 12 months, starting from time of remission until date of censor if the patient did not relapse, or going backwards in time starting from the time of relapse until the time of remission if the patient did relapse. Only full duration timeframes were included.

In each included timeframe, each ANCA measurement was screened whether they fulfilled the criteria of an absolute and relative increase compared to a previous measurement in the past 6 months. The highest slope of these measurements was used as the test variable for the ROC analysis. These were seen as true positive if they occurred in the first timeframe before a relapse and as false negative in any other timeframe. This analysis was performed separately for the ELISA and FEIA method.

We did not employ this method to calculate likelihood ratios because patients had different follow-up durations and therefore different amount of timeframes. Including all timeframes would bias categorical predictors.

Statistical analysis

Numerical variables were expressed as mean (SD) or as median (IQR) and categorical variables as numbers (percentages). Associations were presented as hazard ratios with 95% confidence intervals. A P-value less than or equal to 0.05 was considered significant. All statistical analyses were performed using IBM SPSS statistics for Windows, version 20.0 (Armonk, NY).

To assess the relation of an ANCA rise with a relapse during follow-up, we compared the risk to develop a relapse if a patient had already experienced an ANCA rise or not. We did so by using a Cox regression model with an ANCA rise included as a time-dependent, binary, nonreversible predictor.[13-15] In other words, patients were classified as 0 if a rise in ANCA value had not (yet) occurred and as 1 after the first rise (**figure S1**). An event was defined as a relapse at the time of the start or increase of immunosuppressive treatment. Subjects were censored at the time of last ANCA measurement or death. First, the relationship of an ANCA rise with a relapse was analyzed in the entire cohort as measured by the different ANCA methods. Thereafter, an ANCA rise as measured by antigen-specific solid-phase assays was used to investigate differences in the

relationship of an ANCA rise in subgroups. The cohort was split in subgroups based on renal involvement, diagnosis, induction therapy, ANCA serotype, ANCA pattern and sampling interval.

Results

Receiver operating characteristics curve

Upon dichotomization of the longitudinal data, 547 timeframes were identified, of which 75 prior to relapse and 477 during remission. The area under the curve was 0.723 (standard error 0.037, p<0.001) when analyzing the ELISA and FEIA together. We ran this analysis for the ELISA and FEIA separately to further optimize the cut-off value, although there were only minor differences in the areas under the curve and optimal cut-off values (**figure S3**). We selected the cut-off value that was closest to the upper left corner. The chosen cut-off values for the slope of an ANCA rise as determined by the ELISA and FEIA method were 2.56 and 2.25 %/day, respectively. This is equivalent to a relative increase of 78% and 68% over one month or 233% and 205% over three months.

Different ANCA methods

An ANCA rise as measured by an antigen-specific solid-phase ANCA test was more strongly related to a relapse (HR 5.84 [95%-CI 3.44-9.92]) compared to an ANCA rise as measured by IIF (HR 3.81 [95%CI 2.19-6.63]). The addition of an ANCA rise as determined by IIF did not significantly contribute to the hazard ratio as measured by antigen-specific solid-phase tests for a relapse (**figure S4**).

Maintenance therapy

To assess whether the steroid tapering method influences the relationship between an ANCA rise and disease activity, we categorized patients in either discontinuation (no steroids after 18 months) or tailored (steroids after 18 months) in all patients with a follow-up duration longer than 18 months (n=138). The association of an ANCA rise with a relapse in the different patient groups is shown in figure 1. No notable difference is present in the relation of ANCA with disease activity between patients in whom steroids were discontinued (n=46, HR 4.55 [95%-CI 1.34-15.42], p=0.015) as compared to patients in whom steroids were tailored (n=92, HR 3.81 [95%-CI 1.92-7.56], p<0.001).

After inclusion into our study, 64 of 166 patients did get longer term non-glucocorticoid maintenance therapy (.i.e., more than 18 months +/- 3 months non-glucocorticoid maintenance therapy). Nearly all these patients were included in a clinical study (the REMAIN study or a Dutch randomized clinical trial as presented at the last ANCA workshop in Paris)[16] or were included after

one or more relapses occurring before inclusion into the study. Of the remaining 102 patients 35 patients did also get longer term non-glucocorticoid maintenance therapy because they experienced a relapse within 24 months after the preceding period of active disease. The remaining 67 patients did not get non-glucocorticoid maintenance therapy after 18 (+/- 3 months).

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Method	**Intra-assay variation**	**Inter-assay variation**
ELISA		
PR3-ANCA	4 - 11%	5 - 21%
MPO-ANCA	3 - 12%	4 - 25%
FEIA		
PR3-ANCA	8,2 - 13,9%	12,9% - 18,5%
MPO-ANCA	3 - 9,6%	6,2% - 7,0%

Table S1. Intra- and inter-assay variation of the ELISA and FEIA method for MPO- and PR3-ANCA. The coefficients for the ELISA method were taken from the manufacturers manual and the coefficients for the FEIA method were calculated using our own data.

Sampling Interval	ANCA Pattern	Renal involvement	Induction protocol	Diagnosis	ANCA serotype	Patients (n=166)	Relapses (n=74)	HR (95%-CIs)	P-value
-	-	-	-	-	-	166	74	5.840 (3.440 - 9.917)	**0.000**
<3	-	-	-	-	-	29	5	2.781 (0.390 - 19.820)	0.307
≥3	-	-	-	-	-	137	69	5.061 (2.888 - 8.868)	**0.000**
<4	-	-	-	-	-	69	13	2.131 (0.671 - 6.765)	0.199
≥4	-	-	-	-	-	97	61	6.403 (3.452 - 11.876)	**0.000**
-	PP	-	-	-	-	50	23	3.954 (1.500 - 10.420)	**0.005**
-	NPP	-	-	-	-	116	51	6.615 (3.492 - 12.529)	**0.000**
-	-	Renal	-	-	-	104	44	11.085 (5.005 - 24.551)	**0.000**
-	-	Non-renal	-	-	-	62	30	2.789 (1.302 - 5.976)	**0.008**
-	-	-	Aggressive	-	-	128	49	5.832 (3.003 - 11.325)	**0.000**
-	-	-	Mild	-	-	38	25	6.819 (2.797 - 16.626)	**0.000**
-	-	-	-	GPA	-	126	62	5.526 (2.992 - 10.206)	**0.000**
-	-	-	-	EGPA	-	17	6	2.351 (0.425 - 12.995)	0.327
-	-	-	-	MPA	-	23	6	11.483 (1.958 - 67.363)	**0.007**
-	-	-	-	-	MPO	58	19	4.580 (1.780 - 11.784)	**0.002**
-	-	-	-	-	PR3	108	55	6.286 (3.104 - 12.730)	**0.000**
≥4	-	Renal		-	-	67	39	11.999 (4.870 - 29.564)	**0.000**
≥4	-	Non-renal		-	-	30	22	2.580 (1.005 - 6.622)	**0.049**
-	NPP	Renal		-	-	73	30	14.196 (5.486 - 36.731)	**0.000**
-	NPP	Non-renal		-	-	43	21	2.868 (1.146 - 7.178)	**0.024**
≥4	NPP	Renal		-	-	49	27	11.912 (4.230 - 33.544)	**0.000**
≥4	NPP	Non-renal		-	-	22	16	2.844 (0.959 - 8.500)	0.060
≥4	-	-		-	MPO	29	13	6.618 (2.107 - 20.789)	**0.001**
≥4	-	-		-	PR3	68	48	6.380 (2.915 - 13.963)	**0.000**
-	NPP	-		-	MPO	42	14	10.835 (3.145 - 37.326)	**0.000**
-	NPP	-		-	PR3	74	37	7.516 (2.974 - 18.995)	**0.000**
≥4	NPP	-		-	MPO	21	9	40.071 (3.816 - 420.804)	**0.002**
≥4	NPP	-		-	PR3	50	34	8.186 (2.973 - 22.541)	**0.000**

Table S2. The association of an ANCA rise as measured by the antigen-specific solid-phase ANCA tests with a future relapse in different subgroups of the cohort. A Cox regression model was used and an ANCA rise was included as a time-dependent, binary, nonreversible predictor. ANCA measurement frequency refers to the measurements per year during follow-up, patients were split using a cut-off point of 3 and 4 measurements per year. Patients were defined as persistently positive (PP) when ANCA values never became negative during follow-up and as non-persistently positive (NPP) when an ANCA measurement had at least once been negative. Disease severity was defined according to the WGET Research group.

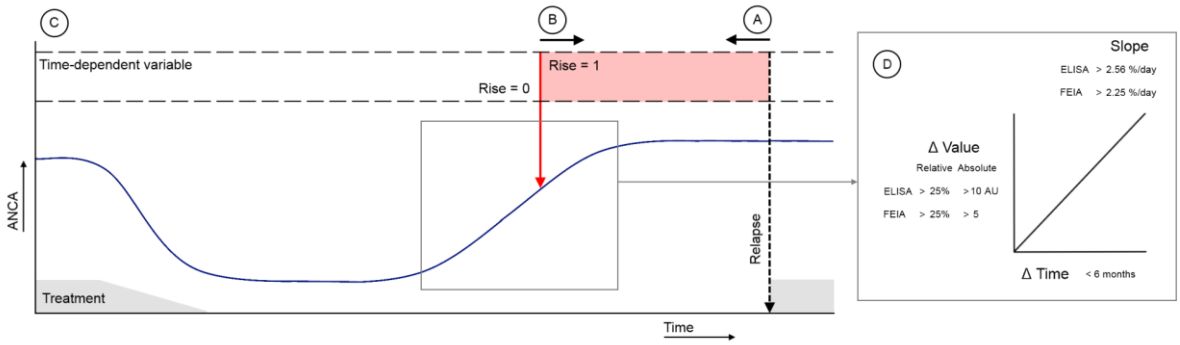

Figure S1. Explanatory picture of the used analysis. The ANCA values of a fictive patient are shown from diagnosis until a relapse. We compared the chance to relapse when a patient had already experienced an ANCA rise or not using a Cox proportional hazards test with an ANCA rise as a time-dependent, binary, nonreversible predictor. In other words, patients were classified as 0 if a rise in ANCA value had not (yet) occurred and as 1 after the first rise. An ANCA rise as measured by the antigen-specific solid-phase assays was defined using the slope, but had to additionally constitute to a relative increase of at least 25% and an absolute increase equivalent to a doubling of the lowest value of a borderline result (at least 10 AU for the ELISA and 5 for the FEIA). The cut-off value for the slope was defined using an ROC curve.

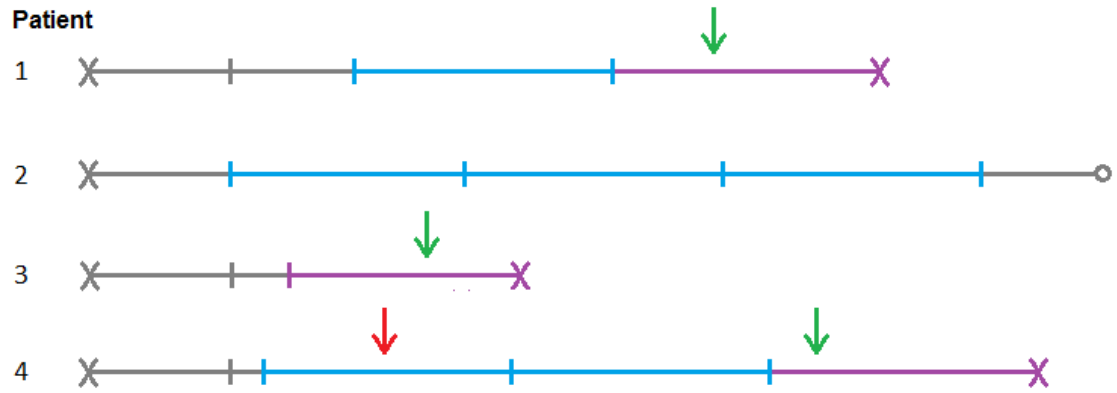

Figure S2. Explanatory figure of the methods used to perform the ROC analysis. Shown is the time to follow-up of 4 fictive patients from their previous disease activity (X) until either a relapse (X) or time of censor (O). The longitudinal follow-up was split in timeframes of a year, the first timeframe before a relapse (purple) was seen as active disease, all other timeframes were seen as remission (blue). Censored follow-up is grayed out. All rises, represented as an arrow, in the first timeframe before a relapse were seen as true positive (green) and as false positive (red) in any other timeframe.

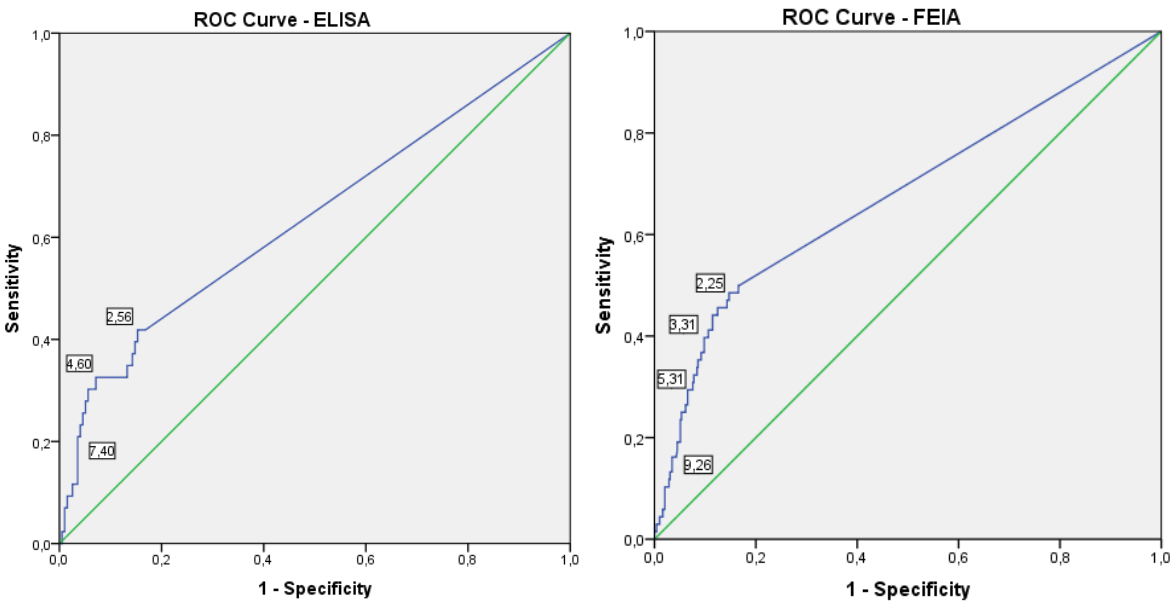

Figure S3. Receiver operating characteristics curves of the slope of an ANCA rise as detected by the antigen-specific solid-phase ANCA methods to detect a relapse in timeframes of 12 months.

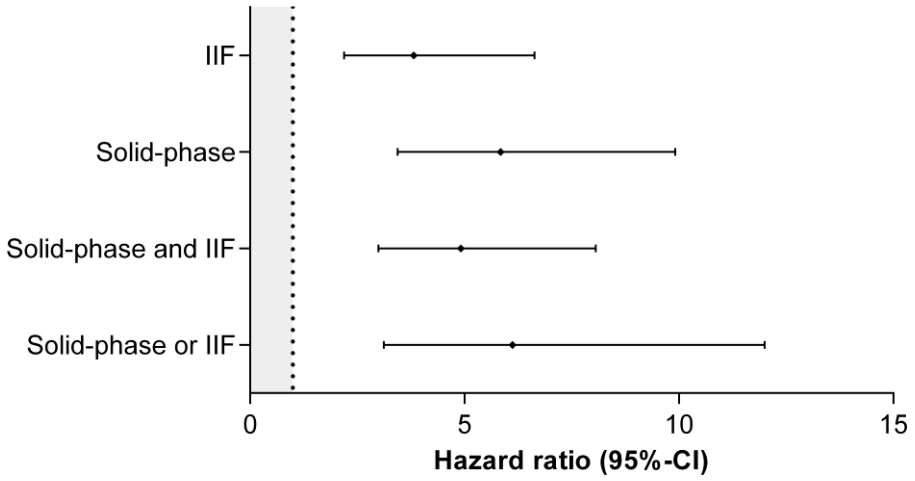

Figure S4. The association of an ANCA rise with a future relapse as determined by several methods in the entire cohort. Hazard ratios are shown with 95% confidence intervals.

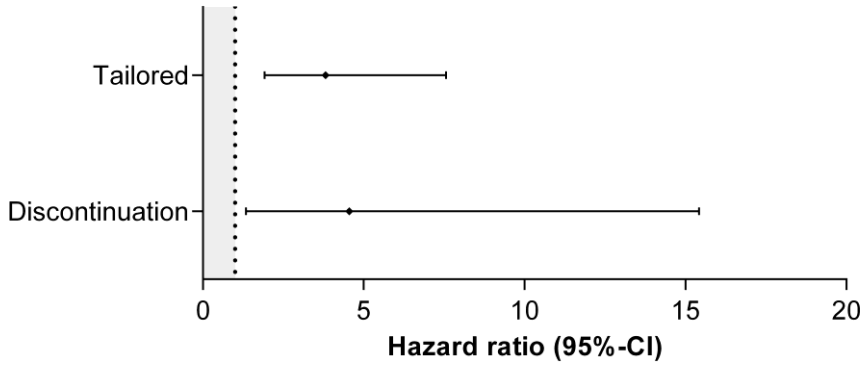

Figure S5. The association of an ANCA rise with a relapse in patients in whom steroids were either discontinued or tailored after 18 months.