ARTHRITIS & RHEUMATISM
Vol. 52, No. 3, March 2005, pp 902–910
DOI 10.1002/art.20938
© 2005, American College of Rheumatology

A Serum Proteomic Approach to Gauging the State of Remission in Wegener's Granulomatosis

John H. Stone,[1] Vinodh N. Rajapakse,[2] Gary S. Hoffman,[3] Ulrich Specks,[4] Peter A. Merkel,[5] Robert F. Spiera,[6] John C. Davis,[7] E. William St.Clair,[8] Joseph McCune,[9] Sally Ross,[2] Ben A. Hitt,[10] Timothy D. Veenstra,[11] Thomas P. Conrads,[11] Lance A. Liotta,[2] and Emanuel F. Petricoin, III,[2] for the Wegener's Granulomatosis Etanercept Trial (WGET) Research Group

Objective. **To identify serum ion patterns that distinguish remission from active disease in patients with Wegener's granulomatosis (WG).**

Methods. **Using sera collected in the WG Etanercept Trial, we selected samples from patients who either were undergoing a period of extended disease remission or had recent flares of active WG. Unfractionated samples were randomized into sets for training and testing, such that remission sera and active disease sera could be analyzed without batch bias. Molecular species within the sera were ionized by high-resolution, matrix-assisted laser desorption ionization time-of-flight mass spectrometry. We then used a bioinformatics pattern-recognition tool to identify optimal combinations of ions. During the training stage, the clinical data (remission versus active disease) were provided in association with the spectral data from each sample. In the testing stage, we performed blinded testing on a previously unexamined set of samples.**

Results. **The most robust model, trained on a total of 82 samples (42 remission, 40 active disease), included 7 key ions with mass:charge ratios of 803.239, 2,171.672, 2,790.574, 3,085.237, 5,051.726, 5,833.989, and 6,630.465. The combined relative amplitudes of these 7 ions identified 5 distinct clusters of either remission or active disease samples during the training stage. In the testing stage, this model segregated 72 samples into the same 5 clusters, including 1 large remission cluster (n = 28) and another large active disease cluster (n = 32). Three smaller clusters of active disease or remission samples were also identified, with remission clusters populated by 2 samples in one cluster and 8 in another, and an active disease cluster populated by 2 samples. The model categorized 35 of 37 remission samples correctly (sensitivity 95%, 95% confidence interval [95% CI] 82.1–99.4) and 32 of 35 active disease samples correctly (specificity 91%, 95% CI 78.1–98.1).**

Conclusion. **This serum proteomic profiling approach appears to be useful in distinguishing between states of stable clinical remission and active disease. Further validation and refinement of this strategy may**

Supported by the NIH (grant N01-AR-9-2240 from the National Institute of Arthritis and Musculoskeletal and Skin Diseases [NIAMS]), the FDA (grant FD-R-001652-01 from the Office of Orphan Products), the National Center for Research Resources/NIH (grants M01-RR0-2719 to Johns Hopkins University School of Medicine, M01-RR0-00533 to Boston University, M01-RR0-0042 to The University of Michigan, and M01-RR-30 to Duke University), Amgen Corporation, Thousand Oaks, CA, and the Maryland Chapter of the Arthritis Foundation (MARRC Program). Dr. Stone's work was supported by NIAMS grant K24-AR-049185-01.

[1]John H. Stone, MD, MPH: The Johns Hopkins Vasculitis Center, Johns Hopkins University School of Medicine, Baltimore, Maryland; [2]Vinodh N. Rajapakse, Sally Ross, Lance A. Liotta, MD, PhD, Emanuel F. Petricoin, III, PhD: National Cancer Institute, FDA Clinical Proteomics Program, Bethesda, Maryland; [3]Gary S. Hoffman, MD: Lerner College of Medicine, Cleveland Clinic Foundation, Cleveland, Ohio; [4]Ulrich Specks, MD: Mayo Clinic, Rochester, Minnesota; [5]Peter A. Merkel, MD, MPH: Boston University School of Medicine, Boston, Massachusetts; [6]Robert F. Spiera, MD: Beth Israel Medical Center, New York, New York; [7]John C. Davis, MD, MPH: University of California, San Francisco; [8]E. William St.Clair, MD: Duke University, Durham, North Carolina; [9]Joseph McCune, MD: University of Michigan, Ann Arbor; [10]Ben A. Hitt, PhD: Correlogic, Inc., Bethesda, Maryland; [11]Timothy D. Veenstra, PhD, Thomas P. Conrads, PhD: National Cancer Institute Biomedical Proteomics Program, Mass Spectrometry Center, Frederick, Maryland.

Dr. Davis has received honoraria (less than $10,000) from Amgen. Dr. Hitt has stock ownership in Ben A. Hitt Correlogic Systems, Inc. and has received patent fees for the Heuristic Method of Classification. Drs. Liotta and Petricoin have received royalty awards from US government–owned patents.

Address correspondence and reprint requests to John H. Stone, MD, MPH, The Johns Hopkins Vasculitis Center, 5501 Hopkins Bayview Circle, JHAAC 1B.23, Baltimore, MD 21224. E-mail: jstone@jhmi.edu.

Submitted for publication July 18, 2004; accepted in revised form December 13, 2004.

help clinicians apply immunosuppressive therapies more judiciously among their patients, thereby avoiding morbidity and mortality from excessive treatment. Identification of the most robust and clinically useful combinations of ions will permit the rational selection of molecules for sequencing and analysis.

Wegener's granulomatosis (WG), one of the most common forms of systemic vasculitis, is the prototype of disorders associated with antineutrophil cytoplasmic antibodies (ANCAs) (1,2). At least 90% of patients with WG sustain permanent morbidity from the disease, including renal failure, pulmonary dysfunction, hearing loss, visual impairment, and chronic sinus problems (1–3). Conventional therapies for WG consist of glucocorticoids and cytotoxic agents such as cyclophosphamide. Complications of these treatment regimens are common and often devastating: in one longitudinal series, 42% of patients experienced permanent morbidity related to treatment (1). Treatment-associated toxic effects include bone marrow hypoplasia, an increased risk of malignancies (leukemia, lymphoma, bladder cancer), infertility, obesity, osteoporosis, hypertension, diabetes, and opportunistic infections. Moreover, despite the currently available therapies, between 50% and 80% of patients experience disease flares after the achievement of remission (1,2,4).

Much of the treatment-related morbidity stems from the difficulty in distinguishing reliably between states of stable clinical remission (which require relatively little or no treatment) and active disease (which require ongoing immunosuppression). Acute-phase reactants usually respond quickly to immunosuppressive therapy, but normalization of these biomarkers is not necessarily predictive of extended periods of disease quiescence. Because the point at which a stable remission has been achieved is difficult to identify, particularly when patients remain on maintenance regimens, there is no nonempirical way of knowing when the toxic conventional therapies may be tapered or discontinued. Significant morbidity results from the use of conventional treatments at higher doses or for longer periods of time than necessary.

Serum proteomic analysis has recently been shown to be an effective means of identifying early or recurrent malignancies in humans with ovarian (5), breast (6), or prostate (7,8) cancer. These approaches have coupled certain types of high-throughput mass spectrometry (e.g., surface-enhanced laser desorption ionization time-of-flight [SELDI-TOF]) with pattern-recognition algorithms. Such strategies have detected significant differences between the serum proteomic profiles of cancer patients and healthy subjects in the low molecular weight range (i.e., <20,000 daltons). The evolution of high-resolution mass spectrometers now permits even greater precision in analyte measurement (9,10). Because systemic vasculitis is a condition in which the target organ (the blood vessel) is in intimate contact with serum, this group of inflammatory vascular disorders is a logical set of diseases in which to test this emerging diagnostic approach.

Using serum samples obtained from WG patients in a randomized, double-blind, placebo-controlled trial (4,11,12), we aimed to identify a set of candidate serum protein patterns (or, more precisely, a pattern of ions) which, through their combined relative amplitudes, can distinguish the sera of patients with active WG from those of patients whose disease is in stable clinical remission. This approach is based on the simultaneous analysis of patterns of ion or peptide fragment combinations, rather than on a preconceived set of molecules known and believed to have utility as biomarkers.

PATIENTS AND METHODS

Source of serum samples. Samples used in this study were collected in the context of the WG Etanercept Trial (WGET) (4) (for a listing of the WGET Research Group, see Appendix A). The design of this trial has been previously described (11). All patients provided informed consent for this study, and all met the modified American College of Rheumatology criteria for the classification of WG (11,13). Etanercept (Enbrel; Amgen, Thousand Oaks, CA) is a soluble fusion protein designed to inhibit tumor necrosis factor. The WGET tested the hypothesis that etanercept is more effective than placebo in maintaining remissions induced by conventional therapy (4,11).

Definitions of disease remission and active disease. The definitions of disease remission and active disease used in this study were identical to those used in the WGET. The WGET Research Group developed and validated the Birmingham Vasculitis Activity Score for WG (BVAS/WG), the instrument used to assess disease activity, in a 1-year study prior to the start of enrollment (14). The instructions manual and practice exercises for the use of the BVAS/WG are available on the Johns Hopkins Vasculitis Center Web site (http://vasculitis.med.jhu.edu). In the BVAS/WG, disease remission is defined as a score of 0, signifying the absence of any new, worse, or persistent symptoms or signs of active disease. In the interest of uniformity with regard to treatment, we selected only patients with severe disease (defined as WG that posed an immediate threat either to the patient's life or to the function of a vital organ [1,11,12,14–16]) and patients who, by virtue of multiorgan system disease, were at risk for the above-defined severe disease.

Table 1. Medications on days of active disease and remission sampling

Medication	No. (%) of samples
Active disease cohort	
No medications	19 (25)
Cyclophosphamide/prednisone	33 (43)
Prednisone	12 (16)
Methotrexate/prednisone	8 (10)
Azathioprine/prednisone	2 (3)
Azathioprine	2 (3)
Methotrexate	1 (1)
Total	77 (100)
Remission cohort	
No medications	35 (21)
Methotrexate	52 (31)
Methotrexate/etanercept	39 (23)
Azathioprine/etanercept	23 (14)
Etanercept	12 (7)
Azathioprine	4 (2)
Cyclophosphamide	1 (1)
Total	166 (100)

Selection of serum samples for study. *Remission samples.* All of the samples selected from periods of disease remission in this study met 2 criteria. First, glucocorticoid therapy had been stopped for at least 3 months prior to the time of sampling (and consequently, all of these patients had been in disease remission for several months prior to sampling). Second, after the sample had been collected, the patients maintained their remission status for at least 3 months. Thus, no patients with disease in remission were receiving glucocorticoids when their samples were obtained, and 21% of the remission samples were collected when the patients were taking no medications at all (Table 1). In accordance with the WGET protocol and standard clinical practice, other immunosuppressive medications were permitted for the maintenance of disease remission. The variety of treatment regimens in both the active disease and remission groups permitted us to evaluate the potentially confounding impact of treatments.

Active disease samples. Samples from patients with active disease were collected either while the patients were receiving no treatment (25% of all active disease samples) or within several weeks after the start of therapy. Because the primary aim of the WGET was to test the efficacy of etanercept in the maintenance of disease remission, patients could be treated for variable lengths of time before entering the trial. All patients with active disease who were selected for this study had either an erythrocyte sedimentation rate (ESR) >50 mm/hour within 2 weeks of serum sampling or a BVAS/WG score ≥3 at the time of study entry.

Regimens for the induction of remission. Among the patients who contributed samples during periods of disease remission, this remission was achieved through a variety of treatment regimens. Because of the nature of severe WG, the majority of remission-induction regimens included cyclophosphamide and glucocorticoids (with or without etanercept, according to the patients' WGET randomization status). The remission-induction regimens included cyclophosphamide/glucocorticoids (47%), cyclophosphamide/glucocorticoids/etanercept (40%), methotrexate/glucocorticoids (4%), cyclophosphamide/glucocorticoids/azathioprine (4%), methotrexate/glucocorticoids/etanercept (4%), cyclophosphamide/glucocorticoids/methotrexate (1%), and cyclophosphamide/glucocorticoids/methotrexate/etanercept (1%).

Acquisition of serum samples. A standard operating procedure outlined in the WGET Manual of Operations was utilized for sample collection. Whole blood was collected in a 10-ml red top tube and allowed to clot for at least 2 hours at room temperature or for up to 24 hours at 4°C. The sample was then centrifuged at 2,000 revolutions per minute (~800*g*) for 10 minutes, and sterile transfer pipettes were used to aliquot at least 1 ml of serum into sterile Sarstedt tubes. Each sample was stored at −80°C.

Chip and sample preparation. Two hundred forty-three samples were prepared using a Biomek 2000 robotic liquid handler (Beckman Coulter, Palo Alto, CA). All analyses were performed using ProteinChip weak cation exchange interaction chips (WCX2; Ciphergen Biosystems, Fremont, CA). A reference sample was applied randomly to 1 spot on each protein array as a quality control for overall process integrity, sample preparation, and mass spectrometer function. The reference sample, SRM 1951A, which is composed of pooled normal human sera, was provided by the National Institute of Standards and Technology (Gaithersburg, MD). Inclusion of this reference sample was designed to identify serum specimens, protein chips, and even individual spots on chips that were not suitable for analysis. Details of the procedures for preparation of the ProteinChip surface have been described previously (17).

Mass spectrometry. Serum mass spectrometry was performed as previously described (5). Briefly, thawed unfractionated serum samples were applied to a WCX2 chip and subjected to mass spectrometry on a QSTAR Pulsar *i* (Applied Biosystems, Framingham, MA). Data were collected without filters and without reduction by peak-picking software. The spectral data streams, composed of peak amplitudes at ~350,000 mass-to-charge (m:z) ratio positions, were exported as .csv files and analyzed by an artificial-intelligence pattern-recognition algorithm (Proteome Quest β version 1.0; Correlogic Systems, Bethesda, MD). The data streams were binned using a function of 400 parts per million. The binning process condensed the number of data points from 350,000 to exactly 7,084 points per sample.

Quality control and assurance. To perform quality control and quality assurance (QC/QA) on the spectra, raw and binned data were plotted against the total ion current (total record count). We examined the mean and SD of the amplitudes, performed chi-square and *t*-test analyses of each ion or bin, and reviewed quartile plotting measures using both JMP software (SAS Institute, Cary, NC) and stored procedures developed in-house. Under this system of QC/QA, spectra that fail statistical checks for homogeneity are eliminated from in-depth modeling and analysis.

Statistical analysis. The first part of the analytic method is a pattern-discovery module that utilizes normalized mass spectral data and combines elements from genetic algorithms (18–20) and self-organizing adaptive pattern-recognition systems (21,22). Genetic algorithms organize and analyze complex data sets as if they were information composed of individual elements, manipulable through a

Table 2. Parameters in the remission and active disease samples in the serum proteomic analysis of Wegener's granulomatosis patients*

	Remission cohort	Active disease cohort
No. of patients	52	66
Mean age, years	53.5	54.6
No. of samples	166	77
Training, no. of samples	42	40
Testing, no. of samples	38	36
Sensitivity, % (95% CI)	95 (82.1–99.4)	–
Specificity, % (95% CI)	–	91 (78.1–98.1)

* 95% CI = 95% confidence interval.

computer-driven process of natural selection. Combinations of ions, randomly selected by the algorithm, are evaluated for their ability to segregate samples from biologic states of interest (in this case, remission versus active disease). The candidate pattern sets are subjected to fitness tests that assess the ability of each pattern to segregate the sera of patients with active disease from those of patients with disease in remission. Patterns that fulfill the fitness test are kept for further analysis and are propagated by mating and recombination with other patterns fulfilling fitness tests. For a more detailed description of the pattern-recognition process, please refer to the National Cancer Institute/Food and Drug Administration Clinical Proteomics Web site (http://clinicalproteomics.steem.com).

Pattern-recognition analyses of the spectra were performed in 2 sequential stages, known as training and testing, respectively. For the purposes of model development and analysis, serum samples were divided randomly into training and testing sets. In the training stage, the clinical data (remission versus active disease status) were provided in association with the spectral data from each sample that comprised the training set. In the testing stage, the model developed during training was used to classify samples that had not been used in the training set. Investigators performing the pattern-recognition analyses were blinded to the classifications of testing-set samples.

During the training stage, the genetic algorithm attempts to identify a limited number of clusters in N-dimensional space, through a survival of the fittest approach. These clusters are plots of Euclidean distance vectors (17), composed of the combined normalized intensities of the randomly sampled m:z values of the remission and active disease samples. During the testing stage, the distance vectors are calculated and plotted for each sample using only the m:z species that comprise the diagnostic model identified in training. N-dimensional plotting yields the classifications of remission, active disease, or neither, depending on whether the sample 1) falls into previously existing remission or active disease clusters formed in training or 2) establishes a new cluster.

RESULTS

Active disease samples compared with remission samples. The 93 patients with WG selected for this study provided a total of 243 serum samples (mean number of samples 2.6, range 1–9). Sixty-six of these patients contributed a total of 77 serum samples from periods of active disease. Fifty-two patients contributed a total of 166 samples from periods of remission. Twenty-eight of the 93 patients (30%) contributed samples from both periods of active disease and periods of remission.

Clinical characteristics of active disease and remission cohorts. All patients who provided remission samples had a history of severe WG before the period of treatment that led to remission. Forty-six of the 66 patients (70%) with active disease were undergoing their first courses of treatment for WG. The mean ages of the patients in the remission and active disease groups were 53.5 years and 54.6 years, respectively. Thirty-seven of the 52 patients with disease in remission (71%) were male, compared with 52 of the 66 patients with active disease (79%) (a male predominance among WG patients with severe disease has been reported previously [12]). Two of the patients with disease in remission (3.8%) and 4 of the patients with active disease (6.1%) were African American. The remainder of the patients in both groups were white, consistent with the known epidemiologic features of this disease (1). The mean BVAS/WG score in the active disease group at the time of serum sampling was 8.3 (range 2–16).

Medications at the times of active disease and remission sampling. The immunosuppressive medications being received by patients in the active disease and remission groups on the day of sampling are shown in Table 1. Similar percentages of patients in the 2 groups were receiving no medications at the time that their samples were obtained (25% of the active disease samples versus 21% of the remission samples; $P = 0.6$). Within the 2 groups, however, patients were receiving a broad range of treatments at the time of sample collection: both the remission and active disease groups were undergoing 7 different treatment regimens. As anticipated, general differences between the treatment regimens of the 2 groups included a preponderance of patients with active disease receiving either the combination of cyclophosphamide/prednisone or prednisone alone (59%), compared with a preponderance of patients with disease in remission receiving either the combination of methotrexate/etanercept or methotrexate alone (54%). There was, however, substantial overlap in the specific types of immunosuppressive medications being taken by patients in the 2 groups (Table 1).

Optimal model. The most parsimonious model, trained on a total of 82 samples (42 remission, 40 active disease) (Table 2), included 7 key ions with m:z ratios of 803.239, 2,171.672, 2,790.574, 3,085.237, 5,051.726,

Table 3. Identification of key ions in the serum proteomic analysis

Key ions	Mass:charge ratio
1	803.239
2	2,171.672
3	2,790.574
4	3,085.237
5	5,051.726
6	5,833.989
7	6,630.465

5,833.989, and 6,630.465 (Table 3). The combined relative amplitudes of these 7 ions identified 5 distinct clusters of either remission samples or active disease samples during the training stage.

In the testing stage, this model segregated the active disease and remission samples into these same 5 clusters, including one large group of samples categorized as active disease (n = 32; cluster 1) and another large group of samples categorized as remission (n = 28; cluster 2). In addition, there were 3 smaller clusters, populated by only 2, 2, and 8 samples, respectively; these clusters were categorized as active disease, remission, and remission clusters, respectively. No new clusters were formed in the testing stage.

The compositions of all 5 clusters with respect to the numbers of remission and active disease samples are shown in Table 4. In the testing set, which included a total of 72 serum samples, this model categorized 35 of 37 remission samples correctly, with a sensitivity for remission of 95% (95% confidence interval [95% CI] 82.1–99.4). The model also categorized 32 of 35 active disease samples correctly, with a specificity of 91% (95% CI 78.1–98.1). The testing-set score results for all 72 samples are summarized in Figure 1.

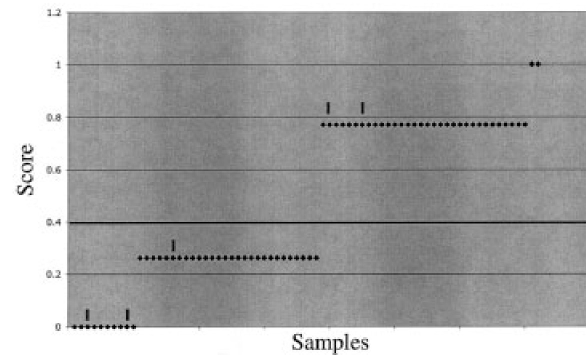

Figure 1. Score results from the test set. Score values, determined by the percentage of homogeneity of each of the clusters formed in training, were calculated for each of the samples in the blinded test set. Each patient sample is represented as a solid diamond on the plot. Correlation between a score and the underlying disease state (remission versus active disease) was determined after unmasking the test results. For this study set, a cutoff score of 0.4 (thick solid line) segregated 94% of the active disease cases and 92% of the remission samples correctly. Vertical bars above the diamonds indicate the spectra that were misclassified by the pattern of key ion values.

Clinical correlations on misclassified samples. Examination of the clinical data on samples that were misclassified within the active disease and remission clusters (n = 3 and n = 2, respectively) revealed that all 3 patients whose samples were categorized by the model as remission samples had been treated with intensive immunosuppression for substantial lengths of time before their active disease samples were obtained. One patient was treated for 25 days with cyclophosphamide (100 mg/day) and prednisone (60 mg/day), the second patient received a 3-day pulse of methylprednisolone (1,000 mg/day) followed by cyclophosphamide (125 mg/day) and prednisone (70 mg/day) for 14 days, and the third patient received cyclophosphamide 150 mg/day and prednisone 60 mg/day for a total of 11 days (Table 4). Although the elevated BVAS/WG scores at the time of randomization reflected disease activity within the 28

Table 4. Distribution of remission and active disease samples within the 5 clusters of the optimal model*

Cluster	Cluster type	No. of samples in cluster (actual distribution)	Comments on misclassified samples
1	Active disease	32 (30 active disease, 2 remission)	One patient's disease flared 12 months after sample retrieval; the other patient became ANCA positive again at the time of sample retrieval, but remains in clinical remission 18 months later.
2	Remission	28 (26 remission, 2 active disease)	The 2 patients with active disease were treated with cyclophosphamide and prednisone for 11 and 25 days, respectively, before serum sampling (see text for details of presampling treatment).
3	Active disease	2 (2 active disease)	
4	Remission	2 (2 remission)	
5	Remission	8 (7 remission, 1 active disease)	The patient with active disease was treated with cyclophosphamide and glucocorticoids for 17 days before serum sampling.

* ANCA = antineutrophil cytoplasmic antibody.

Table 5. Medication regimens of patients who provided serum samples for the large active disease and remission clusters

Medication regimen	No. (%) of samples
Active disease cluster	
No medications	7 (22)
Cyclophosphamide/prednisone	13 (41)
Prednisone	3 (9)
Methotrexate/prednisone	5 (16)
Azathioprine/prednisone	2 (6)
Azathioprine/etanercept	1 (3)
Methotrexate	1 (3)
Total	32 (100)
Remission cluster	
No medications	8 (29)
Methotrexate	8 (29)
Methotrexate/etanercept	5 (18)
Azathioprine/etanercept	3 (11)
Etanercept	1 (4)
Azathioprine	1 (4)
Cyclophosphamide/prednisone/etanercept	1 (4)
Prednisone	1 (4)
Total	28 (100)

preceding days (14), the ESRs of these patients at the time of sampling were normal (17, 17, and 25 mm/hour, respectively).

Two patients with extended clinical disease remission were categorized by the algorithm as having active disease. In both cases, the sample was the first sample (of 4 and 8 consecutive samples from each patient, respectively) obtained in the period of remission. Both of these patients continued to experience clinical remissions for periods of 12 and 18 months, respectively. One of these patients experienced a disease flare 12 months after retrieval of the first remission sample. The other patient became ANCA positive again at the time that his first remission sample was obtained and has remained ANCA positive, but has maintained his clinical remission for 18 months (Table 4).

Sensitivity analyses for medication effects. We conducted sensitivity analyses to ensure that the algorithm did not classify samples merely on the basis of patients' medications at the time of sampling. In the large active disease cluster from the testing set that contained 32 samples, 7 different treatment regimens were included (Table 5). The percentages of samples from patients on each regimen within the large active disease cluster were similar to the percentages of samples from patients on treatment regimens from the overall active disease cohort (compare Table 1 with Table 5). Similarly, in the large remission cluster, the 28 samples from this cluster were from patients on 7 treatment regimens and taking 5 different medications. The percentages of samples from patients on each regimen within this remission cluster were similar to the percentages of samples from patients on treatment regimens within the overall remission cohort (compare Table 1 with Table 5). Thus, it appears unlikely that sera were placed into clusters simply because of the patients' medication regimens at the time of sampling.

DISCUSSION

The current empirical approach to gauging remission in WG leads to substantial morbidity (and mortality) from treatment (1,2,4,23,24). In this study, we describe the development of a serum-based algorithm generated through the use of a pattern-recognition approach. We have demonstrated that a pattern-recognition algorithm trained on WG sera is capable of distinguishing samples from patients whose disease is in remission from those from patients whose disease is active. The optimal model, comprising the combined relative normalized intensities of 7 specific ion species, distinguished between samples from the 2 populations with a sensitivity of 92% and a specificity of 94%. These findings offer a novel approach to biomarker identification that is based on the simultaneous discovery and analysis of a pattern of ions (proteins or peptide fragments) rather than predefined individual biomarkers.

This approach to biomarker identification has a number of potential applications in WG. The fact that patients in the remission cohort had discontinued taking prednisone entirely for several months and had remained off this medication (i.e., remained in clinical remission) for at least 3 months after their samples were obtained indicates that this tool may be an important aid for clinicians, permitting them to know when prednisone can be discontinued successfully. Because it is likely that some patients achieve disease remissions more quickly and with less therapy than others, use of this tool may allow clinicians to individualize patients' length of treatment. Furthermore, through serial monitoring of serum samples, if a patient's serum pattern no longer fits the remission pattern, the algorithm also has the potential to predict disease flares. Both of these potential applications need to be proven in longitudinal, prospective studies.

The field of vasculitis treatment and investigation remains plagued by a paucity of reliable biomarkers. In the late 1990s, the International Network for the Study of the Systemic Vasculitides (INSSYS) performed a longitudinal evaluation of potential biomarkers in 132 patients with systemic vasculitis, including 54 patients with WG. Eleven candidate biomarkers, selected for

their relevance to vascular inflammation, were evaluated. These included von Willebrand's factor, tissue factor, tissue factor pathway inhibitor, thrombomodulin, tissue plasminogen activator, 4 different soluble adhesion molecules (intercellular adhesion molecule 1, vascular cell adhesion molecule 1, endothelial leukocyte adhesion molecule 1, and platelet endothelial cell adhesion molecule 1), C-reactive protein, and the ESR. None of the candidate biomarkers distinguished consistently between patients with active and inactive vasculitis (Hoffman GS: personal communication). Even an elevated ESR, the least specific test in this battery of biomarkers (and yet, the one relied upon by clinicians for decades), had a sensitivity for active disease of only 42%.

ANCAs were not evaluated in the INSSYS study, but these autoantibodies also function poorly as biomarkers. Between 10% and 40% of all WG patients with active disease lack these antibodies altogether (25). Moreover, although persistent ANCA positivity appears to predict an increased likelihood of a disease flare at some point in the future, it does not predict the timing of such a flare and is not a reliable guide to therapy (25,26).

Developments in mass spectrometry, particularly in SELDI-TOF and, more recently, in high-resolution mass spectrometry, offer several substantial advantages over traditional biomarker discovery tools such as 2-dimensional gels (27,28). First, mass spectrometry is the preferred approach to the detection of low molecular weight protein fragments, peptides, and metabolites that are smaller than 10,000 daltons (7,29,30). The group of proteins in this lower molecular weight range have tremendous biologic potential; they contain cleaved or aberrantly shed proteins or peptides that may reflect central features of disease mechanisms (31,32). This information archive is only now beginning to be explored. Second, mass spectrometry offers dramatic improvements in throughput and reduces the quantity of biologic specimens required for analysis. Only microliters of serum are required for the analyses described herein. Third, mass spectrometry approaches can detect tens of thousands of molecules concomitantly, without the need for antibody development and validation.

The approach used in this study also has the advantage of using unfractionated serum. The retention of albumin in unfractionated serum samples may be essential to the diagnostic power of information gleaned from serum through this method. We recently demonstrated that albumin and other carrier proteins of high abundance avidly bind low molecular weight proteins and peptide fragments (33). Such low molecular weight molecules are the underpinning of the diagnostic patterns identified by this approach. Finally, the ease of use of this technique, which requires minimal processing, increases its potential for clinical applications. Identification of individual ions in studies of unfractionated sera is challenging because of the relatively low amplitude of some peaks. For the identification of specific ions, enrichment techniques are usually required.

Studies of the low molecular weight region of the blood proteome are likely to generate many combinations of features with highly accurate diagnostic potential. Such combinations may be identifiable by a variety of pattern-recognition approaches, and the specific identities of ions that comprise discriminatory patterns may yield insights into the pathophysiology of a given disease. The precise identification of the specific ions, an important agenda for future research, remains challenging. Nevertheless, the use of rigorously validated proteomic patterns is already a possibility today in clinical settings, without the absolute requirement for identification of individual ion species. Previous examples of the use of unknown protein analytes prior to definitive identification and sequencing are well established in the medical literature. For example, both CA-125 and prostate-specific antigen, detected by Western blot or radioimmunoassays, were used for many years to aid in the clinical diagnosis of prostate and ovarian cancers, without knowledge of their underlying amino acid sequences (34,35).

Our study has several potential shortcomings. The optimal model did not classify patients' samples correctly 100% of the time, categorizing 3 active disease samples and 2 remission samples incorrectly. As noted, however, all 3 patients whose active disease samples were misclassified had been treated with intensive immunosuppression for 2–4 weeks before their serum samples were obtained. It is possible, therefore, that these patients' sera (and their clinical status) reflected a biology that was actually closer to "remission" than to "active disease." Misclassification may have stemmed from the challenge of characterizing disease activity on the basis of clinical features alone. The misclassification of 2 patients with disease in extended (and subsequently maintained) clinical remissions indicates that this algorithm, like all medical tests, cannot be used in isolation to make clinical decisions. The utility of this algorithm in the prediction of disease flares is the subject of an ongoing followup study.

In conclusion, we have identified patterns of ions within the serum of patients with WG that differentiate the state of remission from the spectrum of active

disease. This serum proteomic profiling strategy is a promising approach for the gauging of disease remission in WG, and therefore addresses a major challenge in the management of this disease.

ACKNOWLEDGMENT

We thank Dr. Philip Seo of Johns Hopkins University for his insightful review of the manuscript.

REFERENCES

1. Hoffman GS, Leavitt RY, Kerr GS, Fauci AS. The treatment of Wegener's granulomatosis with glucocorticoids and methotrexate. Arthritis Rheum 1992;35:1322–9.
2. Reinhold-Keller E, Beuge N, Latza U, de Groot K, Rudert H, Nolle B, et al. An interdisciplinary approach to the care of patients with Wegener's granulomatosis: long-term outcome in 155 patients. Arthritis Rheum 2000;43:1021–32.
3. Seo P, Min YI, Holbrook JT, Hoffman GS, Merkel PA, Spiera R, et al, for The WGET Research Group. Damage from Wegener's granulomatosis and its treatment: prospective data from the Wegener's Granulomatosis Etanercept Trial. Arthritis Rheum. In press.
4. The WGET Research Group. Etanercept in addition to standard therapy in patients with Wegener's granulomatosis. N Engl J Med 2005;352:19–29.
5. Petricoin EF III, Ardekani A, Hitt BA, Levine PJ, Fusaro VA, Steinberg SM, et al. Use of proteomic patterns in serum to identify ovarian cancer. Lancet 2002;359:572–7.
6. Li J, Zhang Z, Rosenzweig J, Wang YY, Chan DW. Proteomics and bioinformatics approaches for identification of serum biomarkers to detect breast cancer. Clin Chem 2002;48:1296–304.
7. Petricoin EF III, Ornstein DK, Paweletz CP, Ardekani A, Hackett PA, Hitt BA, et al. Serum proteomic patterns for detection of prostate cancer. J Natl Cancer Inst 2002;94:1576–8.
8. Adam BL, Qu Y, Davis JW, Ward MD, Clements MA, Cazares LH, et al. Serum protein fingerprinting coupled with a pattern-matching algorithm distinguishes prostate cancer from benign prostate hyperplasia and healthy men. Cancer Res 2002;62: 3609–14.
9. Aebersold R, Mann M. Mass spectrometry-based proteomics. Nature 2003;422:198–207.
10. Conrads TP, Fusaro VA, Ross S, Johann D, Rajapakse V, Hitt BA, et al. High-resolution serum proteomic features for ovarian cancer detection. Endocr Relat Cancer 2004;11:163–78.
11. The WGET Research Group. Design of the Wegener's Granulomatosis Etanercept Trial (WGET). Control Clin Trials 2002;23: 450–68.
12. The Wegener's Granulomatosis Etanercept Trial Research Group. Limited versus severe Wegener's granulomatosis: baseline data on patients in the Wegener's Granulomatosis Etanercept Trial. Arthritis Rheum 2003;48:2299–309.
13. Leavitt RY, Fauci AS, Bloch DA, Michel BA, Hunder GG, Arend WP, et al. The American College of Rheumatology 1990 criteria for the classification of Wegener's granulomatosis. Arthritis Rheum 1990;33:1101–7.
14. Stone JH, Hoffman GS, Merkel PA, Min YI, Uhlfelder ML, Hellmann DB, et al, for the International Network for the Study of the Systemic Vasculitides (INSSYS). A disease-specific activity index for Wegener's granulomatosis: modification of the Birmingham Vasculitis Activity Score. Arthritis Rheum 2001;44:912–20.
15. Sneller MC, Hoffman GS, Talar-Williams C, Kerr GS, Hallahan CW, Fauci AS. An analysis of forty-two Wegener's granulomatosis patients treated with methotrexate and prednisone. Arthritis Rheum 1995;38:608–13.
16. Stone JH, Tun W, Hellmann D. Treatment of non-life threatening Wegener's granulomatosis with methotrexate and daily prednisone as the initial therapy of choice. J Rheumatol 1999;26:1134–9.
17. Hingorani SR, Petricoin III EF, Maitra A, Rajapakse V, King C, Jacobetz MA, et al. Preinvasive and invasive ductal pancreatic cancer and its early detection in the mouse. Cancer Cell 2003;10: 6–21.
18. International Conference on Genetic Algorithms. Proceedings of the Seventh International Conference on Genetic Algorithms. 7th ed. East Lansing (MI): Michigan State University; 1997.
19. Holland JH. Genetic algorithms. Sci Am 1992;267:66–72.
20. Holland JH. Adaptation in natural and artificial systems. 2nd ed. Cambridge (MA): MIT Press; 1992.
21. Lehito P, Kohonen T. Associative memory and pattern recognition. Med Biol 1978;56:110–6.
22. Kohonen T. Self-organization and associative memory. In: Springer-Verlag series in information sciences. Vol. 8. Heidelberg: Spring-Verlag; 1984.
23. Guillevin L, Cordier JF, Lhote F, Cohen P, Jarrousse B, Royer I, et al. A prospective, multicenter, randomized trial comparing steroids and pulse cyclophosphamide versus steroids and oral cyclophosphamide in the treatment of generalized Wegener's granulomatosis. Arthritis Rheum 1997;40:2187–98.
24. Hoffman GS. Treatment of Wegener's granulomatosis: time to change the standard of care? [editorial]. Arthritis Rheum 1997;40: 2099–104.
25. Hoffman GS, Specks U. Antineutrophil cytoplasmic antibodies [review]. Arthritis Rheum 1998;41:1521–37.
26. Boomsma MM, Stegeman CA, van der Leij MJ, Oost W, Hermans J, Kallenberg CG, et al. Prediction of relapses in Wegener's granulomatosis by measurement of antineutrophil cytoplasmic antibody levels: a prospective study. Arthritis Rheum 2000;43: 2025–33.
27. Richter R, Schulz-Knappe P, Schrader M, Standker L, Jurgens M, Tammen H, et al. Composition of the peptide fraction in human blood plasma: database of circulating human peptides. J Chromatogr B Biomed Sci Appl 1999;726:25–35.
28. Paweletz CP, Gillespie JW, Ornstein DK, Simone NL, Brown MR, Cole KA, et al. Rapid protein display profiling of cancer progression directly from human tissue using a protein biochip. Drug Dev Res 2000;49:34–42.
29. Petricoin EF III, Liotta LA. Mass spectrometry-based diagnostics: the upcoming revolution in disease detection. Clin Chem 2003;49: 533–4.
30. Petricoin E III, Liotta LA. Counterpoint: the vision for a new diagnostic paradigm. Clin Chem 2003;49:1276–8.
31. Tirumalai RS, Chan KC, Prieto DA, Issaq HJ, Conrads TP, Veenstra TD. Characterization of the low molecular weight human serum proteome. Mol Cell Proteomics 2003;2:1096–103.
32. Liotta LA, Ferrari M, Petricoin EF III. Clinical proteomics: written in blood. Nature 2003;425:905.
33. Mehta AI, Ross S, Lowenthal MS, Fusaro V, Fishman DA, Petricoin EF III, et al. Biomarker amplification by serum carrier protein binding. Dis Markers 2003–2004;19:1–10.
34. Bast RC Jr, Klug TL, St John E, Jenison E, Niloff JM, Lazarus H, et al. A radioimmunoassay using a monoclonal antibody to monitor the course of epithelial ovarian cancer. N Engl J Med 1983;309:883–7.
35. Stamey TA, Yang N, Hay AR, McNeal JE, Freiha FS, Redwine E. Prostate-specific antigen as a serum marker for adenocarcinoma of the prostate. N Engl J Med 1987;317:909–16.

APPENDIX A: THE WEGENER'S GRANULOMATOSIS ETANERCEPT TRIAL (WGET) RESEARCH GROUP

The institutions and members of the WGET Research Group are as follows: from Johns Hopkins University, John H. Stone, MD, MPH, David B. Hellmann, MD, Lourdes Pinachos, RN, BSN, Michael J. Regan, MD, MRCP, Misty L. Uhlfelder, MPH, Janet T. Holbrook, PhD, MPH, Curtis L. Meinert, PhD, John Dodge, Jessica Donithan, Nancy Min, PhD, Laurel Murrow, MSc, Maria Oziemkowska, MPH, Jacki Smith, Andrea K. Tibbs, BS, Mark Van Natta, MHS, Noel R. Rose, MD, PhD, C. Lynne Burek, PhD, and Monica Taylor, MS; from the Cleveland Clinic Foundation, Gary S. Hoffman, MD, Debora Bork, MFA, Tiffany Clark, CNP, Sonya L. Crook, RN, Sharon Farkas, Sudhakar Sridharan, MD, and Kimberly Strom, CNP; from Beth Israel Medical Center, New York, Robert Spiera, MD, Rosanne Berman, MPH, and Sandy Enuha, MPH; from Boston University, Peter A. Merkel, MD, MPH, Rondi Gelbard, BS, Melynn Nuite, RN, and Aileen Schiller, MS; from Duke University, E. William St.Clair, MD, Nancy B. Allen, MD, Karen Rodin, RN, and Edna Scarlett; from The Mayo Clinic, Ulrich Specks, MD, Kimberly Carlson, Mary Lou Cawson, RN, Medy Clemens, Susan Fisher, RN, Boleyn Hammel, Kathy Mieras, and Steven Ytterberg, MD; from the University of California, San Francisco, John C. Davis, MD, MPH, Maureen Fitzpatrick, MPH, and Steve Lund, MSN, NP; from the University of Michigan, Joseph McCune, MD, Barbara Gilson, RN, Hilary Haftel, MD, Ana Morrel-Samuels, BA, and Sandra Neckel, RN; from Maryland Medical Research Institute, Paul L. Canner, PhD; from Emory University, Doyt L. Conn, MD; from the Arthritis Foundation, Jack H. Klippel, MD; and from the University of Pennsylvania, J. Richard Landis, PhD.