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PII: S0301-472X(18)30849-X
DOI: https://doi.org/10.1016/j.exphem.2018.09.008
Reference: EXPHEM 3676

To appear in: *Experimental Hematology*

Received date: 31 July 2018
Revised date: 18 September 2018
Accepted date: 29 September 2018

Please cite this article as: Valentina Giudice , Angélique Biancotto , Zhijie Wu , Foo Cheung , Julián Candia , Giovanna Fantoni , Sachiko Kajigaya , Olga Rios , Danielle Townsley , Xingmin Feng , Neal S. Young , Aptamer-based proteomics of serum and plasma in acquired aplastic anemia, *Experimental Hematology* (2018), doi: https://doi.org/10.1016/j.exphem.2018.09.008

Highlights

- SOMAscan is an aptamer-based proteomic technology for large-scale studies.
- Nineteen serum proteins are proposed as biomarkers and prognosticators of AA.
- Serum DKK1, SELL, CCL17, and HGF are validated as novel markers of AA.
- Twenty-eight plasma proteins are identified as candidate biomarkers of AA.
- More than 600 proteins can be used as biomarkers of AA in both serum and plasma.

Regular Article

Aptamer-based proteomics of serum and plasma in acquired aplastic anemia

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Running headline: Aptamer-based proteomics analysis in AA

Keywords: aptamers, proteomics, aplastic anemia, biomarkers, bone marrow failure

Category: Genomics/Proteomics of Hematopoiesis

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Abstract: 212 words

Text: 4072 words

Figure Count: 5

Table Count: 1

Supplementary Figures: 8

Supplementary Tables: 9

Reference Count: 51

Clinicaltrials.gov identifiers: NCT00260689, NCT01623167

Abstract

Single-stranded oligonucleotides containing deoxyuridine are aptamers (SOMAmers) that can bind proteins with high specificity and affinity, and slow dissociation rates. SOMAscan, an aptamer-based proteomic technology, allows measurement of more than 1,300 proteins simultaneously for identification of new disease biomarkers. The aim of the present study was to identify new serum and plasma protein markers for diagnosis of acquired aplastic anemia (AA) and response to immunosuppressive therapies (IST). SOMAscan was used to screen 1,141 serum proteins in 28 AA patients before and after therapy and 1,317 plasma proteins in 7 SAA patients treated with standard IST and a thrombopoietin receptor agonist. From our analysis, 19 serum and 28 plasma proteins were identified as possible candidate diagnostic and prognostic markers. A custom immuno-bead-based multiplex assay with 5 selected serum proteins (BMP-10, CCL17, DKK1, HGF, and SELL) was used for validation in a verification set (n = 65) of samples obtained before and after IST, and in a blinded validation cohort at baseline (n = 16). After technical validation, four biomarkers were employed to predict diagnosis (accuracy, 88%) and long-term response to IST (accuracy, 79%). In conclusion, SOMAscan is a powerful tool for identification of new biomarkers. We propose further larger studies to validate new candidate serum and plasma diagnostic and prognostic markers of AA.

Clinicaltrials.gov identifiers: NCT00260689, NCT01623167

Introduction

Acquired aplastic anemia (AA), a bone marrow (BM) failure syndrome characterized by pancytopenia and BM hypocellularity, is caused by hematopoietic stem and progenitor cells (HSPCs) destruction by immune cells [1]. BM transplantation remains the first therapeutic choice for young patients with a matched sibling donor. Immunosuppressive therapies (IST), with or without the thrombopoietin (TPO) receptor agonist eltrombopag (EPAG), are considered the standard of care in older patients, and therapeutic option for younger patients without a matched sibling donor [1-2]. However, the exact mechanisms of action of IST and EPAG are still not well understood [3-5]. Hematologic improvement of blood counts after IST is one of the most supportive indirect evidence of the autoimmunity to HSPC in BM failure [6]. Additional lines of indirect evidence for an immune pathophysiology include measurements of activated cytotoxic T cells that inhibit BM proliferation, circulating and exosomal microRNAs (miRNAs), and the presence of pro-inflammatory cytokines in the plasma [1,7-11].

Measurement of proteins on a large scale in biological samples, or proteomics, has improved slowly compared to other' omics fields [12-13]. Electrophoresis, mass spectrometry, enzyme-linked immune-sorbent (ELISA), and immuno-bead-based multiplex assays are the most utilized techniques to detect and quantify proteins, but large-scale studies often are not feasible due to the limited numbers of samples or analytes that can be studied simultaneously, or technical limitations in the quantification of low abundance proteins [12-15]. An aptamer-based multiplexed proteomic technology, the SOMAscan assay, was released in 2010, and currently between 1,300-5,000 human proteins can be detected simultaneously [15-16]. Aptamers are short DNA or RNA molecules that can bind proteins with low affinity. They are sensitive to nuclease degradation [15], but hydrophobic modifications at the 5-position of deoxyuridine nucleotides greatly increases DNase resistance. Modified single-stranded aptamers, or SOMAmers (Slow Off-rate Modified Aptamers), are tested against targeted proteins from large libraries of randomized sequences through a technique referred to as SELEX (Selected Evolution of Ligands by EXponential enrichment) [13-

15]. Only aptamers with slow dissociation rates (> 30 min) are further selected in order to minimize non-specific binding interactions. As a result, SOMAmers are highly specific for epitopes and residues on many human proteins [13-14]. SOMAmer-target protein complexes are captured by biotin-streptavidin beads, and nonspecifically bound SOMAmers are removed with a polyanionic containing buffer. SOMAmers are then released from their specific target protein complexes by denaturation, hybridized to complementary sequences on microarray, and quantified by fluorescence [13,15]. Using this platform, new biomarkers have been discovered in malignant and non-malignant disorders, such as mesothelioma and Alzheimer's disease [17-18].

In order to broaden current knowledge of proteomics in BM failure, we used SOMAscan to screen serum and plasma proteins from AA patients before and after IST, allowing the identification of new biomarkers of AA and responsiveness to therapy. These proteins may also relate to the overall pathophysiology of BM failure.

Methods

Patients

Sera were collected from 109 AA patients after informed consent was obtained in accordance with the Declaration of Helsinki [19] and protocols approved by the National Heart, Lung, and Blood Institute Institutional Review Board (National Institutes of Health [NIH], Bethesda, MD, USA; Clinicaltrials.gov identifiers: NCT00260689, NCT01623167). All patients were diagnosed as severe AA (SAA) and hematologic response to IST was defined according to standard criteria [20-21]. Patients were divided in three cohorts: a discovery set (n = 28) for large-scale proteomics screening using SOMAscan; a verification set (n = 65) including 21 patients from the discovery cohort for technical validation of selected aptamers by Luminex assay; and a validation cohort (n = 16) of SAA patients whose hematologic response to IST was not known at the time of the study. Specimens were collected at the time of diagnosis and after 6, 12 and/or 24 months of initiating IST. Plasma samples were collected in EDTA tubes from 7 SAA patients (Clinicaltrials.gov identifier: NCT01623167), and specimens were collected at the time of diagnosis and after 6 months of initiating IST and EPAG. Healthy controls were recruited from donors at the NIH Clinical Center Department of Transfusion Medicine. Clinical characteristics are summarized in Table 1. After centrifugation at 2000 RPM for 10 min, serum or plasma samples were collected and stored at -80°C until use.

SOMAscan assay

Large-scale proteomic analysis was performed on serum samples in our discovery set of 28 SAA patients [6 complete (CR), 9 partial (PR), and 13 non-responders (NR)] and on plasma samples from 7 SAA patients (3 CR, 1 PR, and 3 NR) before and after IST by SOMAscan (SomaLogic, Boulder, CO, USA) at the Trans-NIH Center for Human Immunology, Autoimmunity, and Inflammation (CHI), NIH (Bethesda, MD, USA), as previously described [22]. Specimens (50 μL) were diluted to three concentrations (0.005%, 1%, and 40%) to separate groups of high, medium,

and low abundance proteins, respectively, and then combined with dilution-specific SOMAmers. Quality controls (QC) and calibrators, provided by SomaLogic, were run together with internal site QC samples. Data generated from these samples were used to assess inter-assay variability, as reported previously [22].

Luminex validation

For independent validation of candidate serum biomarkers, a custom 5-plex immuno-bead-based multiplex assay was designed based on commercial availability for measurement of bone morphogenetic protein 10 (BMP-10), C-C motif chemokine ligand 17 (CCL17), Dickkopf WNT signaling pathway inhibitor 1 (DKK1), hepatocyte growth factor (HGF), and L-selectin (SELL) in serum or plasma (R&D systems, Minneapolis, MN). Assay was carried out following manufacturer's instructions, and a standard curve and one internal control were included in each plate to reduce inter-assay variability.

Statistics

Data were analyzed using SomaSuite v.1.0.3 (NEC Corporation, Minato, Tokyo, Japan) and web tools developed by the CHI (https://foocheung.shinyapps.io/adat_v02/ and https://foocheung.shinyapps.io/plotterII/) [23]. VENNY 2.1, an interactive tool for comparing lists with Venn diagrams, was used to find common or unique proteins between groups [24]. Unpaired or paired t-tests for two group comparison, analysis of variance (ANOVA) or Kruskal-Wallis test for three-group comparison were performed with Tukey's test for multiple comparison and false discovery rate (FDR) for correction. Pearson correlation was performed using an online tool developed by CHI [25]. Specificity and sensitivity were calculated by receiver operating characteristic (ROC) curves, using the healthy control group as reference [26]. Logistic regression and generalized linear model analysis were performed to calculate the diagnostic and prognostic power of combined markers. By convention, a p value < 0.05 was considered statistically

significant. Principal component analysis (PCA) and t-distributed stochastic neighbor embedding (t-SNE) algorithm for visualization of high-throughput data in two or three dimensions were carried out using RStudio software (v.0.99.896, RStudio Inc., Boston, MA, USA). Protein pathway analysis was performed employing open-source pathway databases [27-28].

Results

Serum proteomic signature of AA

SOMAscan data from the discovery set and a group of healthy controls [n = 14; M/F, 6/8; mean age, 32.3 years old (range, 21-62)] were combined and normalized as previously described [22]. t-SNE of all proteins (listed in Supplementary Table E1) was performed by dividing patients based on hematologic responses to IST at landmark timepoints (Fig. 1A). Although no clear clusters were identified, AA patients, even after treatment, completely separated from healthy subjects, and NR sera appeared different from CR and PR sera after treatment. When t-SNE was performed including blood counts, clearer separations were discerned (Fig. 1B). Next, serum protein levels were compared between healthy controls and AA patients before or after IST by unpaired t-test or compared between patients' groups based on clinical response to therapy (Supplementary Table E2). AA patients' groups before and after IST were compared by unpaired t-test, and proteins higher in each respective group were used to build Venn diagrams (Fig. 1C and Supplementary Table E3). Proteins elevated in sera of CR patients before (n = 7) and after therapy (n = 20) compared to NR were selected as candidate biomarkers of responsiveness to IST. Proteins higher in NR before therapy (n = 15), and in PR before and after IST (n = 2) were selected as candidate biomarkers of non-responsiveness to IST. Among 44 proteins in this group, Wilcoxin Mann-Whitney test with FDR correction was performed to remove proteins showing similar serum levels among groups after treatment, and 19 proteins were selected for further investigations (WISP1, DDR2, FRZB, CNTN4, SELL, THBS1, PDGFA, NID2, HGF, BMP10, TEC, CLEC7A, SGTA, TNFRSF4, PPIF, PRKCZ, CCL17, DKK4, and DKK1) (Fig. 1D). Fifteen out of these 19 proteins were also different in the plasma of AA patients when compared to healthy controls.

As hematological improvements could lead to a subsequent increase in serum proteins due to the appearance of adequate cells in the circulation, candidate protein markers were correlated to blood counts, such as hemoglobin level (Hb), platelets (PLT), absolute reticulocyte count (ARC), absolute neutrophil count (ANC), and absolute monocyte count (AMC) for each patient before and after

therapy (Supplementary Figure E1). Indeed, CCL17, DKK4, DKK1, PDGFA, and THBS1 were highly correlated with blood counts. Multiple correlations also were described for other proteins. As transfusions could also influence circulating protein levels, transfusion history was documented in our cohort of AA patients: 21 of them (2 CR, 7 PR, and 12 NR) had received transfusions before starting IST (mean time between last transfusion and starting drug administration, 144 days; range, 1 day – 60 months). Protein pathway analysis using the Reactome Pathway Database revealed that proteins appeared related to the Wnt pathway, innate and adaptive immune responses, extracellular matrix or cell-to-cell interactions, and hematopoietic stem cell differentiation (Supplementary Table E4). The STRING database also was employed for protein pathway analysis using proteins higher in HC compared to AA patients before IST, or using the selected 19 markers (Supplementary Tables E5-7). Proteins were related to immune response, coagulation, regulation of apoptotic process, regulation of protein phosphorylation, cell adhesion, T cell receptor (TCR), cytokine-cytokine receptor interaction, positive or negative response to cell surface receptor signaling, and Wnt, Ras, HIF-1, NF-κB, and Jak-STAT signaling cascades (Fig. 2).

Validation of candidate serum markers

To assess generalizability of the preliminary SOMAscan findings, a 5-plex immuno-bead-based multiplex assay was applied to a verification set of 65 SAA patients, with samples obtained before IST, at 6 months of treatment, and/or at 1 year after IST. A validation cohort of 16 patients at diagnosis and a group of age- and sex-matched healthy controls [n = 13; M/F, 7/6; mean age, 34.3 years-old (range, 21-62)] also was included. Among the 19 candidate serum markers, proteins linked to the Wnt pathway (DKK1 and BMP10), innate and adaptive immune responses (CCL17 and SELL), and hematopoietic stem cell differentiation (HGF and DKK1) were selected for validation. Serum levels of DKK1, SELL, CCL17, and HGF showed significant correlations between the two techniques (all $p < 0.01$); while BMP-10 serum levels differed between the SOMAscan and Luminex assays ($r = -0.075$, $p = 0.675$). For this reason, BMP-10 was not included

in further analyses. First, SAA patients from the verification cohort were compared to healthy controls, showing all four selected proteins were significantly higher in healthy controls compared to patients (DKK1, SELL, and CCL17, all $p < 0.0001$; HGF, $p = 0.037$) (Fig. 3A). All markers displayed a high specificity for AA [DKK1, area under the curve (AUC) = 0.74; SELL, AUC = 0.89; CCL17, AUC = 0.88; and HGF, AUC = 0.80] (Supplementary Figure E2A). Subsequently, the diagnostic power of combined markers was assessed on verification (AUC = 0.974) (Supplementary Figure E2B) and validation (AUC = 0.832) sets of SAA patients (Supplementary Figure E2C).

Next, SAA patients were divided based on clinical response at 6-month and/or 1-year time points, and groups were compared by one-way ANOVA (Fig. 3B-C and Supplementary Figure E3A). In CR cases, DKK1 was significantly higher at baseline compared to NR patients, and there were increased serum levels also at both 6-month and 1-year time points compared to PR and NR sera. Similarly, CCL17 was higher in CR after IST compared to other groups. No significant differences were present for SELL and HGF. By comparing only CR to NR using unpaired t-test, DKK1 was significantly increased in CR patients ($p = 0.010$), while SELL and CCL17 levels were only slightly higher than those in NR ($p = 0.137$ and $p = 0.132$). No differences were seen for HGF serum levels ($p = 0.389$). Pearson correlation analysis between selected protein markers and blood counts was performed as described above, and multiple correlations were observed (Fig. 3D and Supplementary Figure E3B). Logistic regression and generalized linear model analysis were used to evaluate the prognostic power of combined markers. Data from patients at $\geq$ 1-year follow-up were used to generate a model, and then functions applied to patients from the verification and validation sets at baselines, or at 6 months of therapy (Supplementary Figure E3C-D). For patients at baselines, sensitivity to predict responsiveness to IST was low (48%), while specificity was high (82%); prediction at 6 months of therapy showed higher sensitivity and specificity (76% and 83%, respectively).

Plasma proteomic signature of AA

SOMAscan assay also was employed for screening plasma proteins in a small cohort of AA patients (n = 7) treated with IST and EPAG in order to identify common biomarkers with the serum signature and novel plasma proteins for diagnosis and disease progression. A group of healthy controls (n = 21; mean age, 57 years old; range, 37-62; M/F, 10/11) also was included. Heatmap (Fig. 4A) and PCA were displayed using all 1,317 proteins to visualize a possible signature (Fig. 4B). A proteomic profile of CR patients after IST was compared to those of CR and NR patients before IST. Unpaired t-test with FDR correction (5%) was employed to compare a proteomic profile of healthy controls to that of AA patients; patients' groups before or after IST were compared by unpaired t-test without FDR correction because of the small number of subjects (CR, n = 3; NR, n = 3). In plasma, 600 proteins were different in healthy controls compare to AA, and 35% of them were common to the serum proteomic profile. When plasma protein levels were compared between healthy controls and AA, 43 proteins were present in AA patients' plasma and 43% of them (n = 27) were also present in the serum signature (Supplementary Figure E4 and Supplementary Table E8). In AA patients, 28 proteins were present in CR or NR before IST and could potentially be used as diagnostic and/or prognostic biomarkers of AA (Fig. 4C). After identification of groups of proteins as candidate signatures of responsiveness to IST, heatmaps were displayed using different combinations of those markers. Correlations with complete blood counts were also performed by Pearson analysis (Fig. 4D-E). PCA was carried out using a group of 24 or 60 proteins present in CR or NR after therapy, respectively (Supplementary Figure E5). In both cases, CR patients' plasma proteins were different compared to those of NR patients. These two groups of different proteins were used for pathway analysis; except for complement and coagulation cascades, all pathways in NR cases were common to those found in CR cases (Supplementary Table E8).

Large-scale proteomic profiles obtained from serum and plasma samples of CR patients after standard IST were compared. Because different types of body fluids were screened, we sought common or different proteins between serum and plasma in order to identify a protein signature

independent from the type of body fluid used. More than 80 proteins were present in both serum of patients treated with standard IST and in plasma of CR treated with IST and EPAG. Common proteins were mostly linked to immune response and inflammation, or the extra-/intra-cellular signaling pathways (Supplementary Figure E6 and Supplementary Table E9). Protein pathway analysis was also performed with proteins present only in serum or plasma samples, identifying common pathways. Proteins in serum or plasma were grouped, and PCA was performed accordingly (Fig. 5). Using markers present in serum samples, NR treated with standard IST were different from CR with same treatment, while using proteins present in plasma samples, no clear clusters were visualized. Instead, for patients treated with IST and EPAG, NR and CR were different for both protein groups.

Validation of aptamer-based plasma proteomics profiling

As an indirect method to validate our large-scale plasma proteomic profiling, we investigated plasma levels of circulating cytokines already reported in AA [10]. Although our discovery cohort was small, we confirmed lower CXCL5, CCL5, CXCL11, and EGF plasma levels in our patients at diagnosis (all $p < 0.0001$), and for higher G-CSF and lower CD40 ligand, a similar trend with reported data was also present but did not reach statistical significance ($p = 0.168$ and $p = 0.197$, respectively) (Supplementary Figure E7). In our cohort of treatment-naïve AA patients who received IST and EPAG, TPO was higher in AA patients before treatment ($p = 0.0008$). After therapy, TPO decreased in CR plasma and there were no differences with healthy controls. Circulating c-MPL was also investigated, and AA patients showed persistent lower levels in the plasma and serum (Supplementary Figure E7D). Multiple linear regression was also performed to assess correlation between disease severity and plasma/serum protein levels. Disease severity, defined as the combination of ANC, ARC and PLT count [20], was assessed to serum proteins (DKK1, CCL17, SELL, and HGF) in both serum and plasma discovery sets at baseline

(Supplementary Figure E8). No correlations were described between disease severity and combined markers ($p = 0.083$ and $p = 0.206$ for serum and plasma signature).

Discussion

The discovery of biomarkers has greatly enhanced clinical management of patients with diseases [29-30]. A high-quality biomarker should be informative, sensitive, and specific for the disease, useful in guiding therapeutic strategy, and measurable in a time- and cost-effective manner [15]. Although mass spectrometry remains standard, antibody-based technologies are more sensitive and can detect low-abundance proteins, but multiplexing is limited [15]. SOMAscan assay, an aptamer-based proteomic technology, is 20 times faster than mass spectrometry and applicable to much larger cohort screening [31-33]. Using this platform, thousands of proteins are measured simultaneously with sensitivity and specificity similar to high-quality ELISAs, and large-scale proteomics can be performed for identification of new minimally-invasive biomarkers in malignant and chronic diseases [12-18,33-35]. In this study, we performed for the first time a large-scale aptamer-based proteomic analysis of serum and plasma samples from patients with AA, and many proteins were associated to clinical response to immunosuppressive therapies. Regardless of the body fluid screened, more than 600 proteins were proposed for further validation in larger cohorts as potential biomarkers of AA. In addition, 28 plasma and 19 serum proteins were identified as candidate predictors of responsiveness to IST at diagnosis. From the 19 serum protein signature, 5 candidate markers were selected for validation in a larger cohort of patients (based on availability for Luminex assay). We further examined four new serum candidate markers of AA (DKK1, SELL, CCL17, and HGF) in larger and independent cohorts, showing high specificity for diagnosis (AUC of 0.83 and accuracy of 88%) and long-term response to therapy (accuracy of 79%). Additional candidate markers could be included in this signature based on upcoming analytes available in Luminex. We also propose more than 250 proteins commonly present in both serum and plasma aptamer-based signatures as candidate biomarkers of AA for further validation in larger cohorts. Serum and plasma are not equivalent for analyte measurement (*e.g.* glucose), because anticoagulants and debris alter plasma composition, while cells and platelets release metabolites during clot formation in serum samples [36]. Serum is preferred for low-abundance proteins, while

plasma shows better general reproducibility [36-38]. In our study, 35% of proteins were present in healthy controls in both serum and plasma, and 43% in AA at diagnosis. This diversity in proteomics signature may reflect different composition of these body fluids; however, further studies are needed to better investigate the effects of matrices for biomarker measurement in clinical management of AA patients. Although our study confirms SOMAscan as a powerful and highly specific and sensitive discovery platform for large-scale screening of new biomarkers also in small cohorts [13-14], technical validation of our initial findings is required because SOMAmers recognize conformational epitopes [34].

In addition to technical issues related to data normalization [22], other challenges in assessing high-throughput data are the large amount of information and the need to select proteins linked to the disease itself and not to confounding phenomena, such as drug administration or surgical procedures [33]. In our study, an interactive user-friendly web-based tool was employed for SOMAscan data visualization by PCA or heatmap using pathways listed in the KEGG database, and statistical analysis [23]. The t-SNE algorithm provided better visualization of the high-throughput data than PCA. Because of the possible influence of blood count improvements after immunosuppressive therapy, we rigorously sought confounding in the selection of candidate protein markers by correlations with blood counts using these web-based tools. In particular, DKK1 is expressed by several cell types, such as osteoblasts and osteocytes, but platelets are the major source of circulating DKK1. Circulating DKK1 levels are elevated in diseases in which bone is disrupted (e.g. multiple myeloma), contributing to tumor growth and bone destruction [39-40]. In our cohort, circulating DKK1 levels were decreased in AA patients at diagnosis and in NR after treatment when compared to healthy controls, and correlated with platelet counts. Decreased levels of platelet-related proteins might not only reflect the platelet count but could also influence several biological functions, as platelet-derived DKK1 is involved in macrophage and neutrophil activation and migration during acute inflammation [40-41]. In addition, anticoagulants used for serum collection can cause platelet activation and release of DKK1 [39]. In our cohort of AA patients, we

found circulating DKK1 decreased in both serum (1.5 fold) and plasma (2.3 fold) when compared to healthy controls.

Signatures of responsiveness to IST were extrapolated from our dataset using two independent statistical analyses: two-way ANOVA across all groups before and after therapy; and unpaired t-test with FDR correction (5%) between groups. Proteins that showed differences only after treatment were not included in further analyses, as we believed that these changes might also reflect drug effects and not be predictive of clinical response from baseline. Pursuing this strategy, 19 serum and 28 plasma proteins were selected as candidate biomarkers of AA and disease progression. Here we show that in the current analyses, only one of several possible interpolations of the abundant SOMAscan data was pursued, and we may have failed to identify other biomarkers because of our conservative approach and the small sample size of the discovery cohort.

Protein pathways can disclose known or predicted interactions between selected proteins from open-access databases. For the selected candidate markers, several enriched networks were found, such as Wnt pathway proteins in complete responders. Wnt signaling is important in early hematopoietic ontogeny and maintenance of self-renewing long-term hematopoietic stem cells (LT-HSCs) after stress [42-44]. Conversely, inhibition of Wnt by DKK1 impairs BM recovery after transplantation, reduces LT-HSCs, increases cell cycling, and promotes myeloid compartment expansion [43,45]. Regulation of Wnt is complex and multiple inhibitors and activators are involved [46]. From our signature proteins, four molecules were associated to the Wnt signaling pathway: three are negative regulators (DKK1, DKK4, and FRZB) [45], while WISP1, downstream of WNT1, is increased in many tumors and inflammatory diseases [47]. However, further *in vitro* studies are needed to elucidate the exact role of Wnt-related proteins in the pathophysiology of BM failure.

Some of our candidate markers, such as HGF, also are involved in HSC differentiation [48], and may sustain hematopoiesis together with DKKs during autologous immune attack. From our plasma signature, we confirmed that EPAG overcomes c-MPL blockade by binding the receptor with high-

affinity at a different site [3-4,49-50]. Circulating c-MPL was also investigated, and AA patients showed persistent lower levels in the plasma and serum. Decreased circulating c-MPL in responder patients with normal TPO levels might be due to c-MPL retention on the surface of megakaryocytes and young platelets [51]. However, multiple mechanisms are required to maintain a normal steady state platelet count [51], and free c-MPL could be dispensable as a regulator of thrombopoiesis.

In conclusion, SOMAscan is a powerful tool for large-scale screening of new biomarkers, and the availability of user-friendly software assists in handling high-throughput data in reliable and reproducible. Our initial findings propose some novel candidate biomarkers for diagnosis and disease progression. Further validation in larger and more homogenous cohorts is required, supported by additional experiments. Due to the limited number of commercially available analytes for multiplexed assay, many other possible biomarkers were not examined in our validation analysis. The signature of responsiveness to IST in AA patients might be further improved by pursuing analysis and validation of deeper SOMAscan data.

Acknowledgments

The Authors would like to acknowledge the support of the Trans-NIH Center for Human Immunology, Autoimmunity, and Inflammation (National Institute of Allergy and Infectious Diseases, NIH, Bethesda, MD, USA). This research was supported by the Intramural Research Program of the NIH, National Heart, Lung, and Blood Institute, and by the Trans-NIH Center for Human Immunology, Autoimmunity, and Inflammation.

Authorship

Valentina Giudice, Angélique Biancotto, Xingmin Feng, and Neal S. Young designed the study. Valentina Giudice, Angélique Biancotto, Xingmin Feng, Foo Cheung, Julián Candia, Giovanna Fantoni, Sachiko Kajigaya, and Zhijie Wu conducted the experiments, analyzed the data and interpreted the results. Olga Rios and Danielle M. Townsley collected and analyzed clinical data. Valentina Giudice, Angélique Biancotto, Xingmin Feng, Sachiko Kajigaya, and Neal S. Young wrote the manuscript. All Authors critically reviewed the manuscript content and agreed with the final submission of the manuscript.

Conflicts of Interest Disclosures

The authors declare no competing financial interests.

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Figure Legends

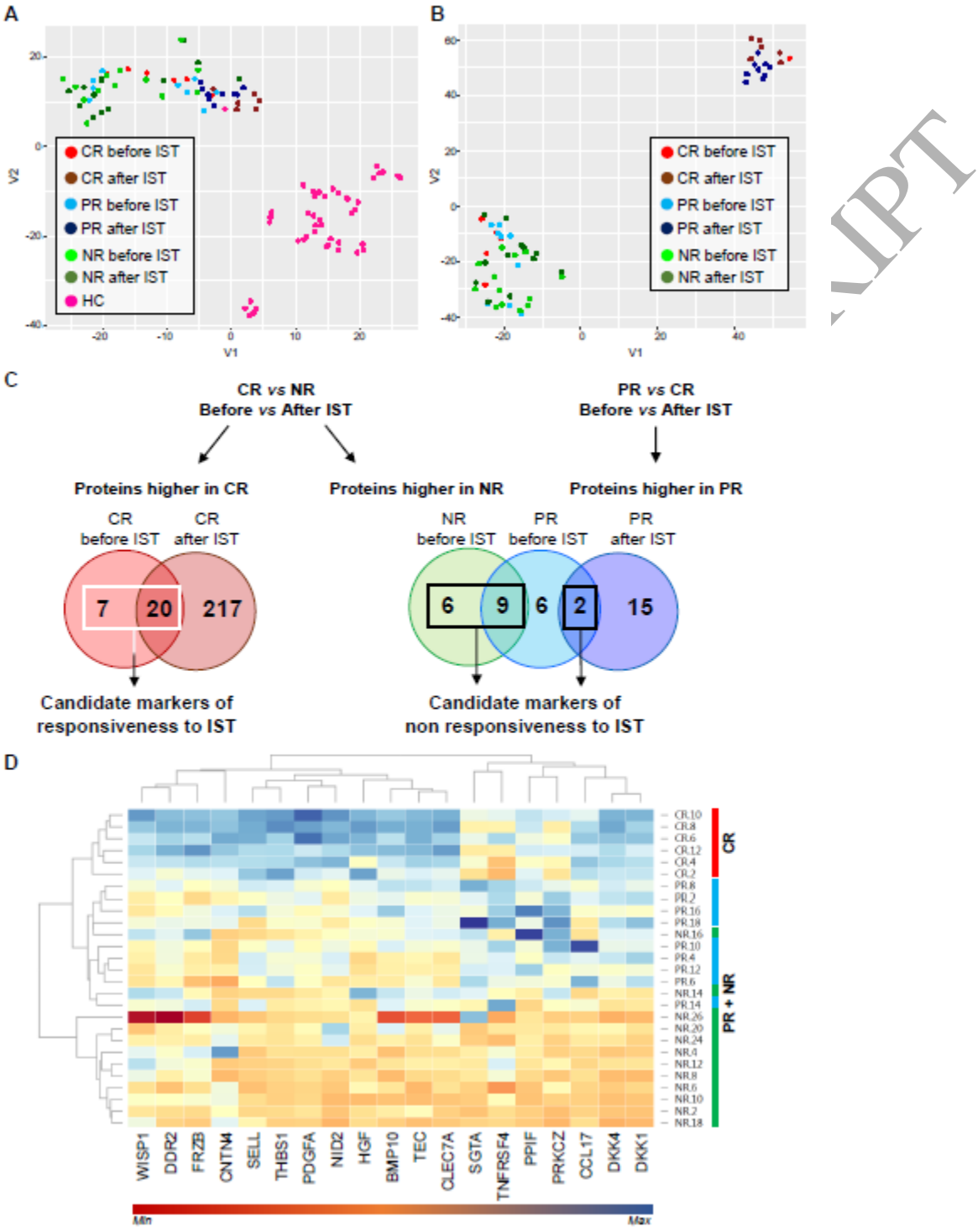
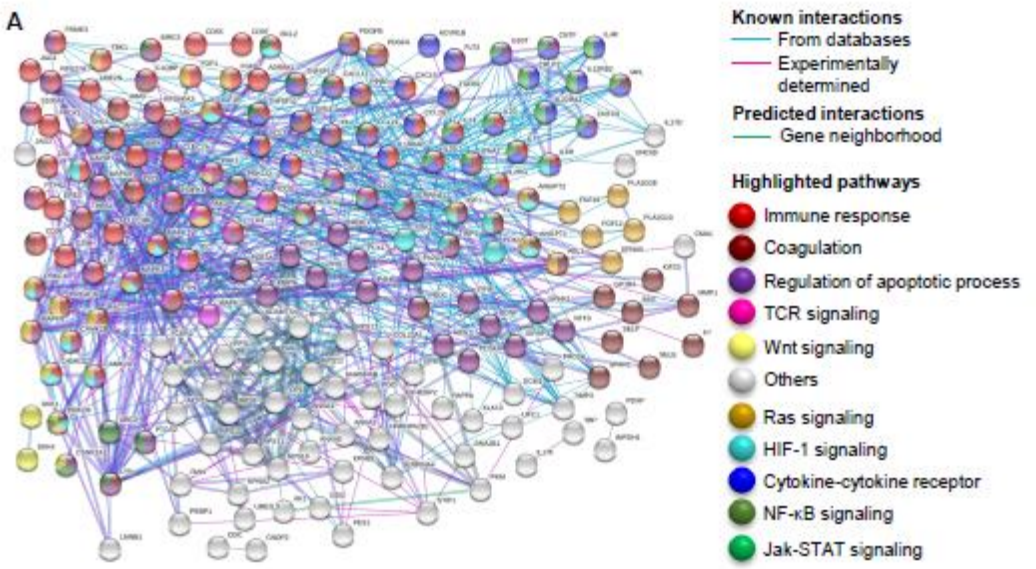
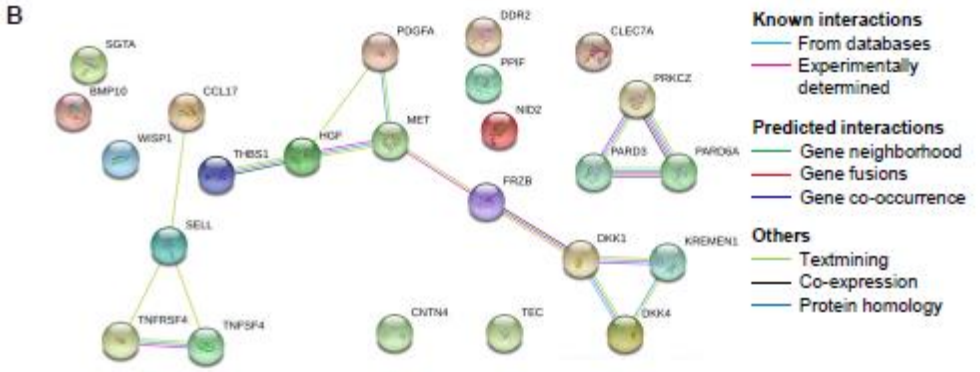


Figure 1. SOMAscan data analysis of serum samples from AA patients. t-SNE testing was carried out on AA patients at diagnosis and after immunosuppressive therapies (IST) and on healthy

controls (HC) (**A**), also including blood counts (**B**). Patients were divided according to clinical response at 6 months or 1 year after treatment: complete (CR), partial (PR), or non-responders (NR). (**C**) In order to find protein markers of responsiveness to therapy, unpaired t-test was performed between CR and NR or PR before and after IST, and proteins were grouped using Venn diagrams. Proteins higher in CR at diagnosis and after IST (n = 27) were chosen as candidate biomarkers of responsiveness to therapy, while proteins higher in NR and PR at baseline and after treatment (n = 16) were selected as candidate biomarkers of non responsiveness to IST. (**D**) Protein levels after treatment of selected 19 candidate biomarkers are shown as a heatmap with hierarchical clustering using a web-based tool. CR patients were grouped together and not mixed with PR or NR.





Top 10 cellular processes in STRING database

Pathway description	FDR	Matching proteins in the network
Cell surface receptor signaling pathway	1.00E-06	BMP10,CLEC7A,DDR2,DKK1,DKK4,FRZB,HGF,KREMEN1,MET,PARD3,PARD6A,PDGFA,TEC,TNFRSF4,WISP1
Regulation of phosphorylation	1.00E-06	BMP10,DDR2,DKK1,HGF,MET,PARD3,PARD6A,PDGFA,PPIF,PRKCZ,TEC,THBS1,TNFRSF4
Regulation of protein phosphorylation	2.11E-06	BMP10,DDR2,DKK1,HGF,MET,PARD3,PARD6A,PDGFA,PRKCZ,TEC,THBS1,TNFRSF4
Regulation of response to stimulus	2.25E-05	BMP10,CLEC7A,DKK1,DKK4,FRZB,HGF,KREMEN1,MET,PARD3,PDGFA,PPIF,PRKCZ,SGTA,TEC,THBS1,TNFSF4
Cell adhesion	4.20E-05	BMP10,CLEC7A,CNTN4,DDR2,PARD3,SELL,THBS1,TNFRSF4,TNFSF4,WISP1
Negative regulation of response to stimulus	4.66E-05	DKK1,DKK4,FRZB,HGF,MET,PARD3,PDGFA,PPIF,PRKCZ,THBS1,TNFSF4
Regulation of cellular protein metabolic process	0.000106	BMP10,DDR2,DKK1,HGF,MET,PARD3,PARD6A,PDGFA,PRKCZ,SGTA,TEC,THBS1,TNFRSF4
Positive regulation of developmental process	0.000106	BMP10,DDR2,DKK1,FRZB,HGF,PARD3,PDGFA,PRKCZ,THBS1,TNFSF4
Negative regulation of cellular process	0.000112	BMP10,CCL17,CNTN4,DKK1,DKK4,HGF,MET,PARD3,PARD6A,PDGFA,PPIF,PRKCZ,THBS1,TNFRSF4,TNFSF4,WISP1
Regulation of branching involved in salivary gland morphogenesis	0.000117	HGF,MET,PDGFA

Figure 2. Protein pathway analysis using serum proteins present in healthy controls (HC) and/or acquired aplastic anemia (AA) patients. (**A**) STRING database was employed for protein pathway analysis using proteins higher in HC compared by unpaired t-test to AA patients before immunosuppressive therapies (IST). Known (from databases or experimentally determined) or predicted (based on gene neighborhood) interactions are shown. Proteins related to immune response, coagulation, regulation of apoptotic process, cytokine-cytokine receptor interaction, and T

cell receptor (TCR), and Wnt, Ras, HIF-1, NF-κB and Jak-STAT signaling cascades are highlighted with different colors accordingly. (**B**) STRING database was employed for protein pathway analysis using the 19 selected serum candidate markers, and the top 10 cellular processes are shown.

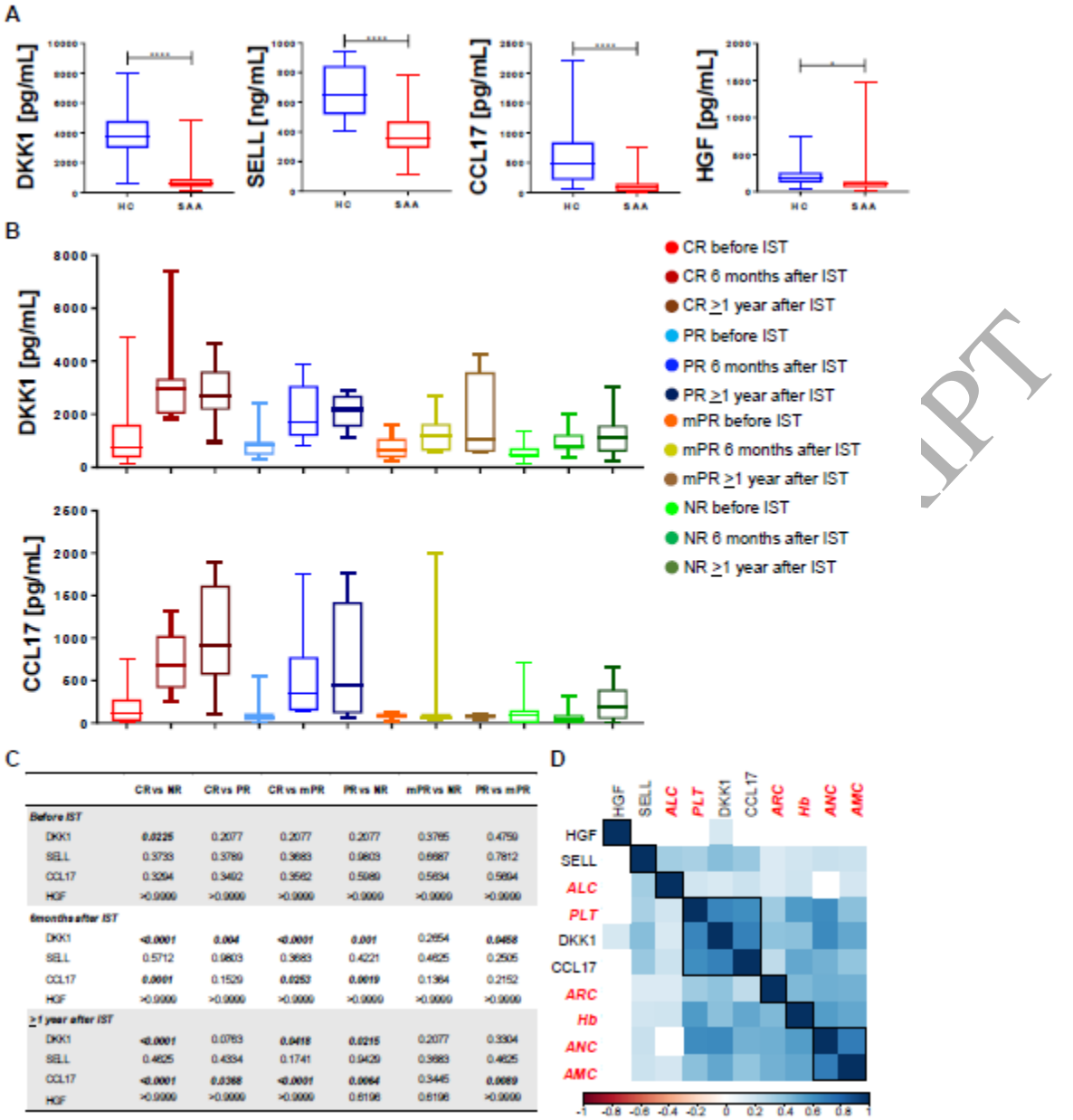


Figure 3. Diagnostic power of selected protein markers in the verification cohort. (**A**) Protein levels were compared between patients and a group of age- and sex-matched healthy controls (HC) by unpaired t-test. Values are shown as minimum to maximum. (**B**) Protein levels were measured by Luminex, and data for DKK1 and CCL17 shown as minimum to maximum for each group of patients. (**C**) Protein levels were compared among groups and timepoints by one-way ANOVA. $p < 0.05$ are bolded. (**D**) Correlation analysis between protein markers and blood counts was performed by Pearson analysis.

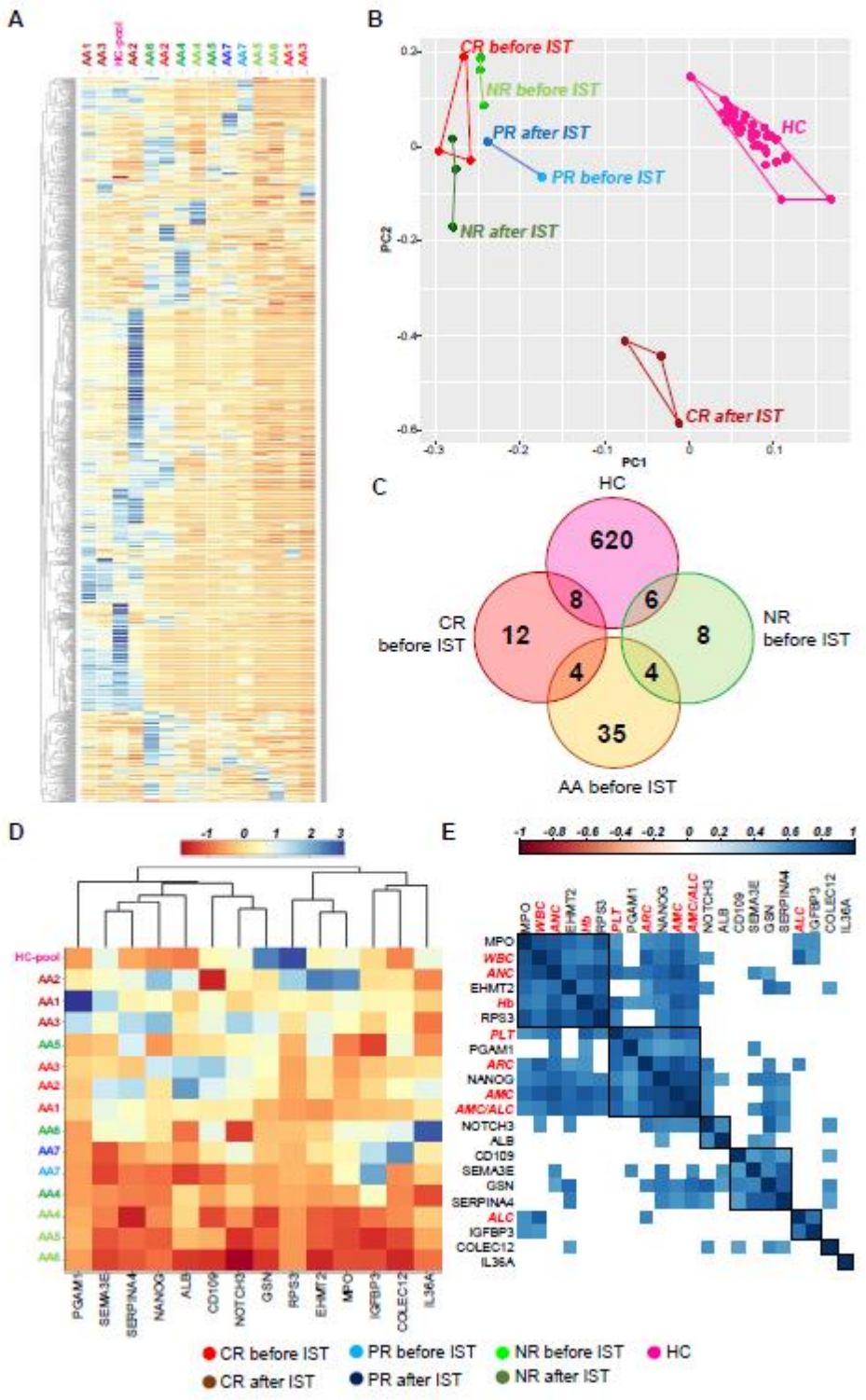


Figure 4. Analysis of SOMAscan data obtained from plasma samples of AA patients. (**A**) Patients were divided based on hematologic response in complete (CR), partial (PR), and non-responders (NR), and heatmap with hierarchical clustering is shown using all 1,317 proteins. Healthy controls were grouped together as a HC pool. (**B**) Principal component analysis (PCA) was also performed

using all 1,317 proteins. (**C**) After unpaired t-test between HC and patient groups, proteins present in each population before immunosuppressive therapies (IST) were grouped and are shown using Venn diagrams. (**D**) Plasma levels of 12 proteins present in CR patients before IST were displayed as heatmap. (**E**) Pearson correlations with blood counts were also performed (blood counts highlighted in red). Correlations with $p > 0.05$ are shown as blank squares. AA, acquired aplastic anemia; WBC, white blood count; ANC, absolute neutrophil count; Hb, hemoglobin levels; PLT, platelet count; ARC, absolute reticulocyte count; AMC, absolute monocyte count; ALC, absolute lymphocyte count.

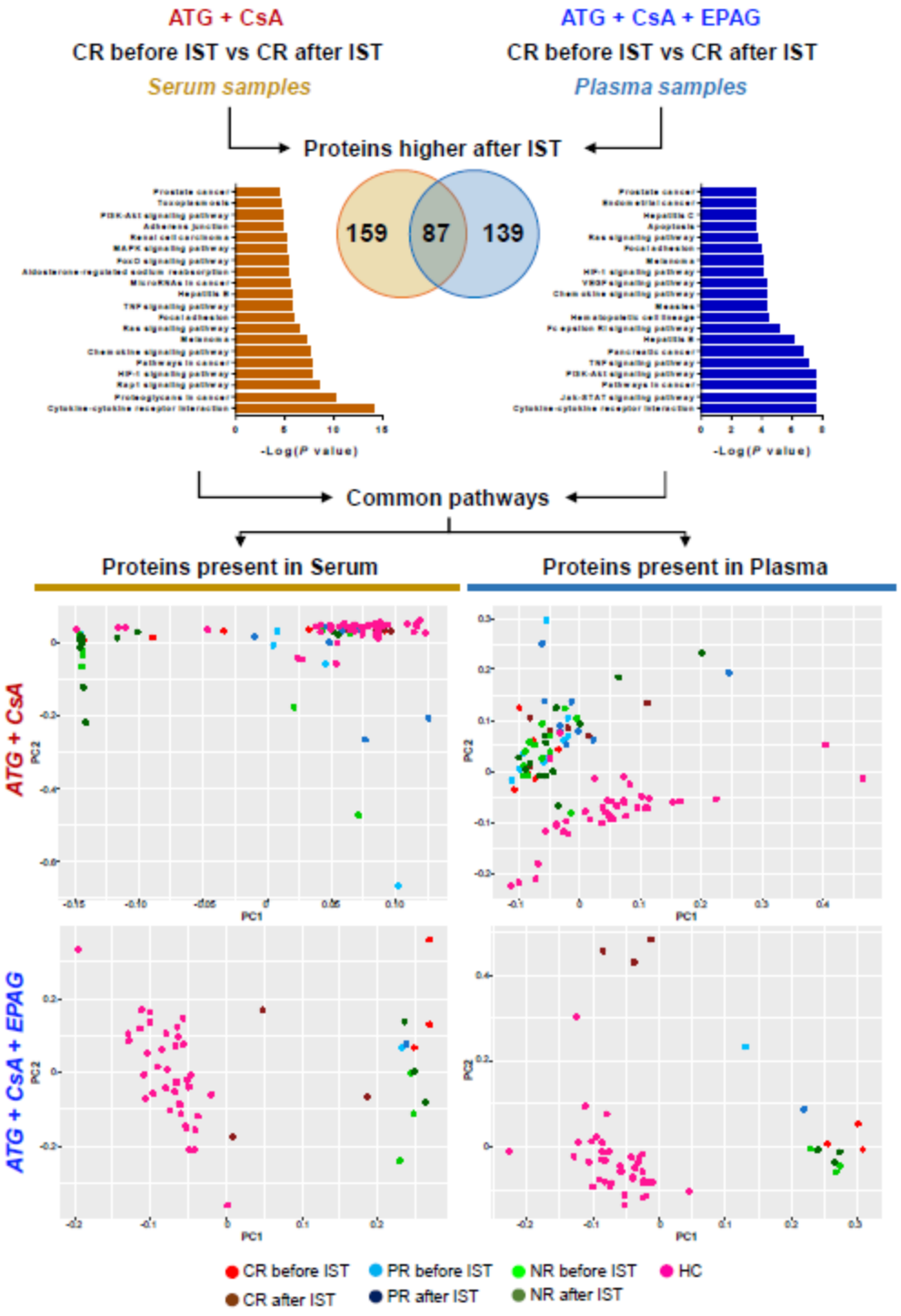


Figure 5. Plasma and serum proteomic signatures in patients after different immunosuppressive therapies (IST). Unpaired t-test was performed between complete responders (CR) before and after therapy using two SOMAscan datasets obtained from serum samples in patients treated with standard IST [anti-thymocyte globulin (ATG) and cyclosporine A (CsA)], or from plasma samples in patients treated with standard IST and eltrombopag (EPAG). Proteins higher in CR after therapy

were grouped and interpolated using Venn diagrams. Using proteins present only in serum (159) or plasma (139), protein pathway analysis was performed, and top 20 pathways are shown (left and right for serum and plasma proteins, respectively). Multiple common pathways were identified, and proteins from serum are plasma signatures were grouped. Using these two groups of proteins, PCA was performed in patients treated with standard IST (upper row) and in those who received standard IST and EPAG (bottom row). Patients were divided according to hematologic responses to therapy in complete (CR), partial (PR), and non-responders (NR). HC, healthy controls.

Supplementary Figure E1. Correlations of candidate serum markers with blood counts**.** As blood count improvements after IST could be a confounding parameter in biomarker selection, Pearson correlation analysis was performed using a web-based tool. Pairwise complete observation was chosen for handling NA values, and hierarchical clustering was applied. Correlation with a $p > 0.05$ are not displayed. Blood counts are shown in red: ALC, absolute lymphocyte count; ARC, absolute reticulocyte count; ANC, absolute neutrophil count; AMC, absolute monocyte count; Hb, hemoglobin; PLT, platelet count.

Supplementary Figure E2. Receiver-operating curve analysis for specificity and sensitivity and diagnostic power of selected markers for AA diagnosis. (**A**) Sensitivity and specificity were calculated by a receiver-operating curve analysis, using the healthy control group as a reference. AUC, area under the curve; CI, confidential interval. A combined protein marker panel was used to predict the diagnosis of SAA in the verification (**B**) or validation (**C**) cohorts using the HC group as reference. AUC, area under the curve; CI, confidence interval; *$p < 0.05$; ****$p < 0.0001$.

Supplementary Figure E3. Serum levels of candidate biomarkers in the verification cohort and their correlations with blood counts. (**A**) Protein levels were measured by Luminex, and data for HGF and SELL are shown as minimum to maximum for each group of patients. (**B**) Correlation

matrix used to build correlograms in Fig. 4. By using all four markers, a generalized linear model analysis was calculated on patients at 1-year follow-up and used to predict responsiveness to IST at baseline (**C**), or after 6 months of treatment (**D**). CR, complete responders; PR, partial responders; mPR, minimal partial responders; NR, non-responders; IST, immunosuppressive therapies; ALC, absolute lymphocyte count; PLT, platelets; ARC, absolute reticulocyte count; Hb, hemoglobin levels; ANC, absolute neutrophil count; AMC, absolute monocyte count; AUC, area under the curve.

Supplementary Figure E4. Common proteins in serum and plasma samples from aplastic anemia (AA) patients before immunosuppressive therapies (IST). (**A**) Unpaired t-test was performed in serum or plasma proteins between healthy controls (HC) and AA patients before therapy. From this analysis, 478 or 16 proteins were present in serum or plasma of AA patients, respectively, and 27 were common in both signatures. Using this group of 27 markers, a heatmap with hierarchical clustering (**B**) and Pearson correlations with blood counts (**C**, highlighted in red) are displayed. Correlations with $p > 0.05$ are shown as blank squares. WBC, white blood count; ANC, absolute neutrophil count; Hb, hemoglobin levels; PLT, platelet count; ARC, absolute reticulocyte count; AMC, absolute monocyte count; ALC, absolute lymphocyte count.

Supplementary Figure E5. Plasma proteomic signature in patients after immunosuppressive therapies (IST). (**A**) After unpaired t-test between HC and patient groups, proteins present in each population after IST were grouped and are shown using Venn diagrams. PCA was performed using 24 proteins present only in CR patients (left panel), or 60 proteins in NR subjects (right panel). Protein pathway analysis was carried out using these two groups of proteins, and top 20 pathways in CR (**B**) or 16 pathways in NR (**C**) are shown.

Supplementary Figure E6. Serum and plasma protein pathway analysis in complete responder (CR) patients after IST. Unpaired t-test in serum and plasma proteins was performed between complete responders (CR) before and after therapy as described in Figure 2. Proteins higher in CR after immunosuppressive therapies (IST) were grouped and interpolated using Venn diagrams. Using proteins present both in serum and plasma (87), protein pathway analysis was performed, and top 50 pathways are displayed. ATG, anti-thymocyte globulin; CsA, cyclosporine A; EPAG, eltrombopag.

Supplementary Figure E7. Validation of cytokine signatures in plasma. Heatmap with hierarchical clustering (**A**) and Pearson correlations with blood counts (**B**, highlighted in red) are shown using cytokines already reported in aplastic anemia (AA). (**C**) Data are also reported as mean+SD in healthy controls (HC) and AA patients before or after immunosuppressive therapies (IST). Cytokine expression was compared between AA patients and HC by unpaired t-test. (**D**) Plasma levels of TPO and free c-MPL are reported as mean+SD in healthy controls (HC) and complete (CR) or non-responder (NR) patients before or after immunosuppressive therapies (IST). Groups were compared to the HC by one-way ANOVA with Dunnett's multiple comparisons test. WBC, white blood count; ANC, absolute neutrophil count; Hb, hemoglobin levels; PLT, platelet count; ARC, absolute reticulocyte count; AMC, absolute monocyte count; ALC, absolute lymphocyte count; RFU, relative fluorescence unit. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, ****, $p < 0.0001$.

Supplementary Figure E8. Multiple linear regression analysis between disease severity and combined markers. Multiple linear regression analysis between disease severity and validated biomarkers (DKK1, CCL17, SELL, and HGF) was performed by RStudio. Disease severity was defined as the combination of absolute neutrophil count (ANC), absolute reticulocyte count (ARC), and platelet (Plts) count. Linear regression was assessed in plasma (A) and serum (B) samples using corresponding discovery sets. For each analysis, Residuals vs Fitted plot for non-linear patterns,

Scale-location plot for variance visualization, Normal Q-Q plot for normal distribution, and Residuals vs Leverage plot for influential outlier identification are shown. Statistics is reported on the right of each panel. *, $p < 0.05$.

Table 1. Patients' characteristics

	Serum			Plasma
	Discovery set	Verification set	Validation set	Discovery set
	n = 28	n = 65	n = 16	n = 7
Median age, years (range)	30 (10-65)	33 (2-75)	47 (9-78)	36 (7-65)
Sex (M/F)	18/10	37/28	7/9	3/4
Treatment				
ATG+CsA	28	56		
Epag+ATG+CsA	-	9	16	7
Clinical response				
NR	13	31	3	3
PR	9	20	6	1
CR	6	14	3	3
Relapse/unknown	-	-	4	
Baseline CBC				
Median ANC (cells/µL)	392 (0-1430)	485 (0-1881)	1103 (0-4700)	500 (20-1190)
Median ALC (cells/µL)	1170 (290-2691)	1358 (137-3243)	1244 (370-2580)	1300 (360-2620)
Median AMC (cells/µL)	104 (0-250)	123 (0-393)	226 (0-1470)	100 (10-240)
Median ARC (10^3 cells/µL)	18.275 (2.3-45.8)	27.809 (1-130)	38.044 (6.6-105.7)	36.8 (7.2-65.1)
Median Hb (g/dL)	7.7 (5-11)	8.6 (5.4-13.7)	9.4 (7.2-13.2)	8.3 (7.6-9.3)
Median Platelet count (/µL)	13750 (1000-78000)	26497 (1000-229000)	57375 (12000-209000)	33300 (17000-59000)
Post-treatment CBC				
Median ANC (cells/µL)	1339 (30-3260)	1262 (70-3260)	-	1300 (320-2790)
Median ALC (cells/µL)	1038 (260-1910)	1120 (9-3162)	-	1300 (570-2580)
Median AMC (cells/µL)	302 (64-640)	307 (10-1310)	-	200 (50-560)
Median ARC (10^3 cells/µL)	42 (1-97)	49.55 (2.9-153)	-	58.2 (9.8-143.6)
Median Hb (g/dL)	10 (7-15)	10.6 (7-16.1)	-	10.4 (7.2-15)
Median Platelet count (/µL)	62536 (6000-231000)	72989 (1000-346000)	-	70400 (6000-181000)

ATG, anti-thymocyte globulin; CsA, cyclosporine; Epag, eltrombopag; NR, non-responders; PR, partial responders; CR, complete responders; CBC, complete blood count; ANC, absolute neutrophil count; ALC, absolute lymphocyte count; AMC, absolute monocyte count; ARC, absolute reticulocyte count; Hb, hemoglobin.