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Systematic evaluation of plasma signaling cascades by functional proteomics approaches: SARS-CoV-2 infection as model

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Clinical Significance

Acute phase proteins constitute plasma signaling cascades of great importance and interest in clinical diagnosis. Although they are well-known and defined proteins in clinical practice, their physiological behavior (changes in absolute or relative abundance depending on the pathology) makes it difficult to establish profiles and associate them in order to stratify patients or differentiate between pathologies. In a disease as critical as COVID-19, any effort to achieve a better diagnosis of the patient is a great advance. In this work, a large number of acute phase proteins have been simultaneously determined and correlated with the severity of patients infected with SARS-CoV-2 virus. A new design of a multifunctional platform has allowed us to learn more about how these proteins behave in plasma and to value their potential as a clinical tool.

Abstract

Purpose: Acute phase reactants play a critical role in inflammation. The difference in their physiological functions or the different dynamic ranges of these proteins in plasma makes it difficult to detect them simultaneously and to use several of these proteins as a tool in clinical practice.

Experimental design: a novel multiplex assays has been designed and optimized to carry out a high-throughput and simultaneous screening of acute phase reactants, allowing the detection of each of them at the same time and in their corresponding dynamic range.

Results: Using Sars-CoV-2 infection as a model, it has been possible to profile different patterns of acute phase proteins that vary significantly between healthy and infected patients. In addition, severity profiles (acute respiratory distress syndrome and sepsis) have been established.

Conclusions and clinical relevance: Differential profiles in acute phase proteins can serve as a diagnostic and prognostic tools, among patient stratification. The design of this new platform for their simultaneous detection paves the way for them to be more extensive use in clinical practice.

Introduction

Inflammation is a non-specific biological process in response to tissue damage mediated by different stimuli, either external, such as the entry of pathogens (infection or sepsis), or by persistent tissue damage. Inflammation is commonly observed in pathologies such as cancer, autoimmune diseases, or exposure to chemical and biological agents. Symptoms of inflammation include pain, blushing of

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the skin and fever, among others. These signs respond to hemodynamic changes such as increased vascular permeability or the accumulation of immune system cells in the damaged and/or infected area. In a more molecular sense, the relationship between the inflammatory response and the immune system is well defined. Inflammation is encompassed within the innate immune response [1-3].

As indicated, tissue damage leading to the inflammatory response can be originated from pathogen infection. Cell death caused by pathogens develops a "danger" signal that stimulates the innate immune response through pathogen-associated molecular patterns (PAMPs). The interaction of PAMPs with receptors on immune cells triggers an alarm signal that promotes phagocytosis by activation of adaptative immune system (antigen-presenting cells and CD8+ and CD4+ T cells) [4]. In addition, the inflammatory response may also be triggered by the response to tissue damage. Programmed cell death has been considered a "silent" process in terms of immune system activation [5]. However, it has been shown that under certain circumstances of stress, cells can develop a pro-inflammatory process characterized by the release of danger-associated molecular patterns (DAMPs), culminating in increased T-cell activation. This phenomenon has been termed immunogenic cell death [6] and can generate a complete immune response because of cell damage, for example, cell death in response to certain anti-tumor therapies [7,8].

In addition to activation of the humoral immune system, hemodynamic changes associated with the inflammatory response respond to well-characterized physiological processes such as complement activation, coagulation, or fibrinolysis [9]. These processes are characterized as a network of plasma signaling cascades that interconnect with each other and have effects at systemic levels. In the clinical practice, these proteins are known as acute phase reactants (APRs) or proteins [10].

APRs are those plasma proteins that display altered levels increase or decrease in blood during an inflammatory process, thus speaking of positive (increase) or negative (decrease) APRs. These are wide variations, being rom twice the normal values to 1000-fold the basal concentrations. Another outstanding characteristic lies in the different dynamic ranges in which these proteins are found in plasma. For example, albumin presents normal plasma values of around 4 g/100 mL while for C-reactive protein normal values are below 1 mg/100 mL [10]. According to this relative plasma abundance, the simultaneous multiplex detection of APRs represent a challenge in several pathological situations. Despite the knowledge of the physiological role of APRs in inflammation, there is a lack of both adequate techniques and standard results. Thus, these signaling cascades as a whole are not used as biomarkers for diagnosis or treatment of patients. In routine analyses only some specific parameters such as C-reactive protein, serum amyloid A or some coagulation factors are assessed [11,12]

In addition, the profile of these APRs in patients might be correlated with the severity of the disease/infection [3] or in the success of vaccination by establishing the inflammatory response before the doses [13]. Also, these group of proteins play a different role in homeostasis during infection such as: Activation of phagocytosis (C-reactive protein); antithrombotic activity (α1-glycoprotein) or inhibition of inflammatory response (α2-macroglobulin) [3,12,13]. Hence, deciphering significant profiles and levels fluctuation -of APRS proximal human fluids- is highly relevant in these pathologies.

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The severe coronavirus disease 2019 (COVID-19) caused by severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) has been shown to be a localized pulmonary infection characterized by the presence of acute respiratory distress syndrome (ARDS) and has also had systemic implications with generalized sepsis in the most critical patients [14].The pathological features of ARDS are diffuse alveolar damage manifested by alteration of the capillary interface, as well as accumulation of immune cells (innate and adaptive) and protein-rich exudates in the alveolar spaces [15] Patients with COVID-19 have elevated levels of cytokines and various inflammatory mediators, such as IL-1β, IL-6, IFN-γ or TNF-α, in lung proximal fluids and peripheral blood. The release of this cytokine storm has been attributed to SARS-CoV-2 infection, eliciting localized inflammatory responses in the lung area but also systemic [16](Figure 1).

Sepsis is defined as life-threatening organ dysfunction caused by a dysregulated patient response to infection. This condition is associated with an in-hospital mortality of more than 10 % [17]. In the case of SARS-CoV-2 infection, the process of sepsis-induced coagulopathy (SIC) has been observed, a vascular pathology which manifests itself in the early stages of the disease and which courses with thrombosis, but which has not been so severe in patients as to generate hemorrhages, although it does seem to be responsible for the vascular sequelae of this disease [18]. In patients with COVID-19, the increase in proinflammatory factors in response to unknown antigens and tissue damage in turn releases DAMPs that contribute to an increase in the innate immune response and AFP in the patient. Some of these DAMPs such as HMGB1 possess a coagulant effect that aggravates the dysregulation of vascular homeostasis to the development of disseminated intravascular coagulation (DIC), which is characterized by thrombi in the microvasculature and is associated with multiorgan dysfunction and hemorrhage [19].

In relation to acute phase proteins and their relationship with this process, evidence has been found of significant changes in plasma concentrations of some of them in SARS-CoV-2 infection. Albumin, C-reactive protein and serum amyloid A are PFAs that are routinely studied in the clinical analysis of hospitalized patients. Data from these PFAs have been analyzed and taken as biomarkers in COVID-19 disease, proving to be of interest for monitoring pathology or determining severity. Other clinical parameters such as prothrombin time or partial thromboplastin time have been taken as indicators of patient status [20,21].

Protein microarray allows the miniaturization of thousands of immunoassays in a single device, evaluating many biomolecules presented in one biological sample with high-sensitivity and reproducibility with a small amount of sample [22,23]. Moreover, protein microarrays have been reported as useful methodology to analyze differential protein profiles of study samples by antigen-antibody interaction [24,25]. In the lack of a feasible methodology for determining all the APRs in one single step, in this report, it is explored protein microarrays as a suitable tool to carry out a high-throughput and simultaneous screening of APRs, allowing the detection in their corresponding dynamic range.

These customized protein microarray has been evaluated to decipher differential APRs profiles in COVID19 disease as it is an infection with different stages where inflammation is involved in the evolution and satisfactory resolution of the infection.

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Material and Methods

Materials

All reagents were of analytical grade and were used as received without further purification. Isopropyl alcohol, ethanol 96 %, 3-(2-Aminoethylamino)-propyldimethoxymethylsilane (MANAE) (≥95.0 %), BS3 (bis(sulfosuccinimidyl)suberate), dimethyl sulfoxide (DMSO), glycerol 85 %, bovine serum albumin (BSA), Tween™ 20, Hybriwell sealing system, Grace Bio-Labs ProPlate® microarray system, Grace Bio-Labs ProPlate® clips for microarray systems (Sigma-Aldrich, St. Louis/MO, USA); SuperBlock™ Blocking Buffer (TBS), Pierce™ BCA Protein Assay Kit., EZ-Link NHS-PEG4 Biotin, Blocker™ BSA (10 %) in PBS, Microscope slides (76 x 26 mm), Mseries Lifterlip y Lifterslip™ coverslips, Thermo Scientific, Rockford/IL, USA); Microarray-Specific 384-well Microplates, JetStar™, Optimum Microarray Printing Buffer C (ArrayJet, Roslin, UK); Corning® 96-well Black Flat Bottom Polystyrene Not Treated Microplate (Corning, Somerville, Massachusetts, USA); Cytiva Amersham™ Streptavidin-Fluor Cy3 (GE-HEALTCARE, Little Chalfont, Buckinghamshire, UK). APRs tested in microarray: Albumin (ALB), serum amyloid A (SAA), serum amyloid P (APCS), thrombin (THBR), ceruloplasmin (CP), von Willebrand factor (VWF), complement factor C3 (C3), complement factor C4 (C4A), factor VIII (F8), ferritin (FTH1), fibrinogen (FGA), haptaglobin (HP), mannose-binding lectin (MBL2), plasminogen (PGA), C-reactive protein (CRP), retinol transporter protein (RET4), prothrombin (F2), transnferrin (TF), transcortin (CBG), transthyretin (TTHY), α-1-antichymotrypsin (CTRC), α 1-antitrypsin (A1AT), α-1-acid glycoprotein (ORM1) and α-2-macroglobulin (A2M). Antibodies employed in this study are detailed in Table S1.

Patients and Samples.

Cohort 1: Plasma samples from 20 patients diagnosed with COVID-19 by RT-PCR and 10 healthy donors (COVID-19 negative) were collected in the University Hospital of Salamanca (HUS, Salamanca, Spain). Cohort 2: plasma samples of 72 COVID-19 patients confirmed by RT-PCR obtained from PROTEORED-ISCIII and collected from patients during their hospital stay. Detailed information about patients of both cohorts studied is this report is collected in supplementary Table S2.

1.1 Equipment and software

ArrayJet® Printer Marathon v1.4, JetSpyder™ 12 samples, JetStar™ (ArrayJet, Roslin, UK); Scanner SensoSpot Fluorescence (Miltenyi Imaging GmbH, Radolfzell, Germany); Orbital shaker (FALC Instruments S.r.l.; Treviglio, Italy); Fisherbrand™ Microplate Vortex Mixers (Fisherbrand™, EEUU); T100 Thermal Cycler (Biorad, Hercules/CA, USA); Digital Dry Block Heater (Thermo Scientific, Rockford/IL, USA); GenePix® Pro Microarray Analysis Software (Molecular Devices, San Jose/CA, USA); R statistics software (R Foundation for Statistical Computing, Vienna, Austria. http://www.R-project.org/).

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1.2 Detection of acute-phase proteins (APRs) by affinity proteomics

1.2.1 APRs array design

Based on previous reports [24], protein array content was designed with different antibodies (Table S1) targeting different APRs. Firstly, stock solution from commercial antibodies was adjust to a concentration of 1 µg/µL. Then, each antibody was resuspended in PBS diluted at 5 different concentrations (1:100, 1:500, 1:1000, 1:5000, 1:10000 v/v) with respect to the stock concentration. Subsequently, antibody aliquots were diluted 1:1 (v/v) in Arrayjet Printing buffer C, according to ArrayJet Printer Marathon v1.4 specifications. Slide-out has 7 identical subarrays with 432 spots, each antibody is printed in triplicate. Positive (Cy3, anti-biotin antibody and biotin) and negative (GST-antibody, PBS, clean buffer, and printing buffer) as controls were also included in each subarray. A total of 6 serum samples were analyzed per array. Antibodies were deposited on a chemically activated surface prepared according to previous reports [18] with ArrayJet Printer Marathon v1.4. Eventually, printed arrays were packed and stored protected from light in dry atmosphere at room temperature (RT) until assayed.

Sera Biotinylation.

Following the protocol described previously by Henjes F et al.[19], plasma proteins (100 µg) were biotinylated by incubation with 0.78 mg/mL of NHS-PEG4-biotin (12 µL in DMSO) for 2 hours at 4 °C. Biotinylation reactions were stopped with 0.5 M Tris-HCl (pH 8) obtaining a final concentration of biotin 1:200 (v/v) per serum sample. [27,28]

APRs screening

Firstly, 100 µL of biotinylated serum 1:100 (v/v) in SuperBlock® BSA were prepared. Epitope retrieval was performed according to a previously described method [29]. Microarrays were blocked and washed with distilled water (3 times, 5 min). Then, samples were incubated overnight at 4 °C with mild shaking. After that, the arrays were washed with PBS with Tween (PBST) (0.05 %) (3x, 5 min) and revealed using 1:200 (v/v) Cy3-Streptavidin for 30 min. Finally, arrays were washed with PBS (3x, 5 min) and distilled water, dried and scanned.

Image acquisition

Array images were obtained by Scanner SensoSpot Fluorescence. The TIFF images generated by array scanning were analyzed using GenePix Pro 6.0. software. Parameters were set to quantify light intensity values at Cy3 (λ 532 nm) [23,28].

2.7 Bioinformatics Analysis

2.7.1. APRs and AAB microarray data pre-processing and quality control

The fluorescence signal retrieved from images processed in the previous section was corrected by subtracting background signal and then transformed into Z score as previously reported [30,31]. Overall raw fluorescence and $\log_2$ (Z score) density distribution were compared to validate signal correction at each microarray employed. Principal Component Analysis (PCA) was performed to

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discard any microarray-wise batch effect (Figure S1). Data processing and analysis were performed in R environment [32]

2.7.2. Biostatistics and Data visualization

Volcano plots illustrate the statistical significance of Z score ratio changes at any two defined conditions. Z ratio is calculated by subtracting the mean Z score in condition A and mean Z score in condition B and then dividing it by the overall standard deviation of Z score in conditions A and B as previously described [33]. Volcano plot Y-axis represent the statistical significance of Z score mean difference in condition A and B -Wilcoxon Rank Sum test, - $\log_2$ (p-value)-. Canonical biplot is a visualization technique extensively applied to interpret Principal Component Analysis (PCA). The biplots presented in this work draw both observation and variables -patient samples and microarray proteins respectively- as dots and directed vectors. The vector size and direction indicate the discriminatory power of protein variables at the first two Principal Components. Importantly, the direction of vector variables at biplot can also reveal correlations between sets of protein variables and therefore, corroborate the trends observed in Volcano plots. The plots presented in this work were generated using *ggplot2*, *ggpubr, ggbiplot, Epi, ComplexHeatmap* and *pathview* R packages [34-39]

Results

Here, it has been designed and developed a customized antibody microarray in order to perform the simultaneous detection of APRs and to determined global profiles of selected panel of APRs. This new multiplex strategy has enabled to decipher specific APRs profiles associated to SARS-CoV-2 patients. Concurrently, high-throughput analysis has also deciphered protein patterns to stratify the severity of the pathology.

Design and development protein microarray for detection differential APRs profiles.

The wide dynamic range of APRs in plasma is a challenge for biomarkers seeking in plasma; among that mostly of APRs display increment or diminutions of relative abundance in plasma, which is directly correlated with the pathological situation or evolution (acute phase, chronic phase…). Bearing this in mind, it has been explored to optimize critical features of the protein arrays in order to screen in one single step this panel of selected APRs. Then, antibody deposition has been studied in order to increase the yield of immobilization [25,26,39]. Also, target capture has been evaluated at different antibody dilutions (1:100, 1:500, 1:1000, 1:1000, 1:5000 and 1:1000 v/v).

Subsequently, the immobilization of the antibodies on the activated surface of the microarray is controlled by two different adsorption strategies; which have been compared based on depositions

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rate: i.-Fast deposition at low temperature (lyophilization, freeze-drying process); ii.-Slow deposition at constant temperature (37 ºC) for long period of time (10 days) (Figure 2A). The differences between both adsorption strategies are easily observed according to spot morphology and differences in spot signal (Figure 2).

Comparing the number of positive spots in the two types of immobilizations (Figure 2B), a weaker overall signal was observed in the microarray obtained by fast deposition than slow deposition rate. Among the differences in spot number, it is observed a similar trend regarding spotted antibody concentration, where the high number of positive spots is obtained a high antibody concentration. When analyzing the spot signal distribution in each microarray (Figure 2C), a normal distribution of the signal was observed in the immobilization methodology at low deposition rate, which contrasts with the bimodal distribution obtained in the global spot signal at high deposition rate. Furthermore, this last trend observed is homogeneous in all the dilutions tested in this study.

Bearing in mind these results, it seems that the freeze-drying methodology (fast deposition) presents fewer uniform results and lower sensitivity than the methodology than low deposition rate (at 37ºC). Taking into consideration the differences between the two methodologies, a hypothesis has been formulated about the behavior of the antibodies deposited on the microarrays (Figure 2D). In the case of freeze-drying, where the temperature drops and the following sublimation is performed rapidly, could lead to an uneven and disordered immobilization of the antibodies on the spot, not allowing all antibodies to be bound directly to surface of the microarray. Subsequent incubation with biological samples involving changes in pH and osmolarity could lead to the desorption of antibodies not direct attached to the surface. Thus, smaller spot sizes are obtained. On the other side, the immobilization process at constant temperature allows a more uniform spatial arrangement of the antibodies over time, allowing all of them to adsorb on the surface of the microarray. The adsorption of all the deposited antibodies corresponds to a spot with larger diameter where the sensitivity increases and can be considered more uniform as mostly of the spotted antibodies seem to be attached to the array surface.

On the other hand, observing the trend in which positive spots are presented, the lower the concentration, the lower the number of positive spots. This is maintained until the lowest concentration of 1:10.000 is reached. In view of the results, it seems reasonable to discard at least the lowest concentration for the subsequent study of the APRs, in order to avoid false positives in the detection of the proteins.

Evaluation of differential APR profiles

Once the most appropriate immobilization method for the antibodies on the microarray surface was evaluated, the feasibility to determine differential profiles between groups of patients was systematically explored. After incubation of the serum samples, the signal obtained for each protein (at different antibody dilutions) was analyzed in detail and a comparison was made between control individuals and COVID-19 positive individuals. It is observed a certain tendency which discriminate between individuals +/- COVID-19 disease (Figure 3A). Based on the results of the antibody concentrations for each protein, it was observed that the 1:5000 (v/v) concentration was the most

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sensitive for discriminating significant differences between protein concentrations of control individuals versus individuals SARS-CoV-2 infected (Figure 3B and S1). This fact together with the results of the immobilization process make us determine that this concentration will be the one to be used for the analysis of this and other APRs studies as developed in the following section of the report. Furthermore, in the overall comparison of the APR results at that concentration a certain trend can be observed in the decrease of APR globally in COVID-19-positive individuals.

In order to further investigate the analytical capabilities of this newly developed method, the sensitivity and specificity of the method have been analyzed. For this purpose, binomial logistic regression models were generated to evaluate the discriminatory power of the APR protein matrix to classify healthy patients and patients with COVID-19. The figure 4 shows receiver operating curve (ROC) analysis (AUC =0.8) with 5 proteins: A1AT, F8, FGA, HP and MBL2.

Determination of biomarkers of severity in SARS-CoV-2 infection.

After optimization and stablish conditions for fine-tuning of the methodology for the customized array for APRs detection, this array has been used to decipher biomarkers of severity in SARS-CoV-2 infection, searching for differential patterns of APRs ьbetween individuals with different clinical characteristic. The most severe complications of COVID-19 disease are ARDS and sepsis [40,41]. The first approach of the data was performed by comparing infected SARS-CoV-2 individuals which developed ARDS as part of their pathology (Figure 5A). In this analysis, there is a certain trend in the studied panel of APRs across individuals +/- ADSR.

Sepsis is another of the most serious complications of SARS-CoV-2 infection. In this regard, it is interesting to analyze whether there are different APRs profiles when sepsis is of pulmonary origin or if the initiating factor of sepsis is tissue damage in another body site (extrapulmonary sepsis) (Figure 5B). Regardless of the origin of sepsis, patients with COVID-19 disease can present ARDS, the correlation between both variables may be of interest to determine whether there is a profile APRs that can determine the progress or not of ARDS when sepsis is present.

Detailed analysis of the APRs profiles between the different clinical groups in the patient cohort resulted in significant differences in several APRs profiles (Figure 5C). When comparing the APRs profiles in patients with extrapulmonary sepsis, several APRs could be observed to have significant differences between individuals +/- ARDS. When this analysis was similarly performed in individuals with sepsis of pulmonary origin, the number of proteins with significant differences was lower, although the variation in change appears to be greater in this comparison group (Figure 5D). These results correlate with those obtained in the first cohort studied, where significant results had also been observed. Both cohorts present a decrease in APR with the development of ARDS and present commonly detected proteins in both studies.

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Discussion

The novel customized APR microarray has successfully employed to detect in different dynamic ranges of a selected panel of APRs with clinical relevance. Thus, demonstrating the success of the methodology for massive and multiparametric studies of biomolecules with low sample quantity. In addition, with the design and development for the fast and simultaneous determination of the APRs, it has been possible to establish differential protein profiles for SARS-CoV-2 infection and for the different severity of the disease. Also, from a methodological point of view, the dynamic design of this microarray also allows the inclusion of other proteins that may be of interest in COVID-19 disease, other infections or diseases that involve variations in APR patterns such as cancer [42] or autoimmune diseases [43].

In the general, the observed APRs profiles, in the two studied, display a tend to decrease with the course of the disease (Table 1). As in any infection, the overcoming of the epithelial and endothelial barriers that serve as the organism's first defense activates the innate immune system by interaction with the PAMPs giving rise to the inflammatory process. On the other hand, persistent tissue damage by infection and the release of DAMPs also has their impact on the activation of the immune system which is reflected in an increment in APRs [44]. The processes of systemic inflammation or sepsis respond with a high activation of acute phase protein cascades. It might seem that the decrease of the antigenic stimulus or the repair of tissue damage could lead to a rapid recovery of the levels of this panel of APRs. However, it has been shown that APRs can remain chronically activated after prolonged sepsis or conversely, sepsis processes can lead to periods of immunosuppression after persistent inflammation [45].

The immunosuppression associated with COVID-19 patients has been described in the most recent literature where a significant reduction in circulating T lymphocytes, although a high concentration of immune system cells and proinflammatory factors in the pulmonary area, which would be correlated with the appearance of ARDS [46]. Likewise, the phenotype of T lymphocytes found in patients with COVID-19 has been shown to present the typical markers of "exhausted" activated leukocytes (CD25, HLA-DR, PD-1), which hinders a satisfactory immune response [47]. Despite these parameters, B-lymphocyte activity was not affected, as seen in the high production of Igs observed in this work, which has also been reported in other studies correlating ARDS with the immune response of patients [48].

As an added value to the study, these results suggest the possibility of establishing more personalized treatments for each type of patient if a more in-depth study of APRs is carried out. Considering the plasma cascades collected in content of this customized microarray (Table 1), it can be observed that, in patients with sepsis of extrapulmonary origin, the variations in plasma levels of APRs originate in the different cascades and subgroups that make up the APRs set. In the case of pulmonary sepsis, it seems that the most significant variations are more focused on the coagulation cascade. The treatments indicated in patients with COVID-19 are based on preventive anticoagulant therapies such as heparin and the use of corticosteroids such as dexamethasone as anti-inflammatory drugs [49,50], which agrees with the pattern of APRs described in this work. However,

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the administration of drugs with more specific targets on some APRs could lead to an improvement in the treatment of severe cases.

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Related with the profiling of APRs, it is necessary to point out that this platform is not a quantitative approach. As it was described in the introduction, APRs are proteins whose plasma levels range from mg/dL to g/dL [10]. Considering this intrinsic characteristic of APRs as a whole, the most interesting application of our novel platform is to detect variations at the same time of all the proteins regardless the dynamic range. In this sense, comparing with mass spectroscopy used for the most abundant APRs, our novel method provided the possibility of exploring all the proteins in one single assay with low amount of sample and in a relative short time, testing more than 1000 analytes in just one array [22]. According to our previous experience in novel microarrays, we also have established a complete pipeline for development and analysis from the platform design to the final results useful for clinical outcome [23,30,31].

Overall, in our study we found a decrease in APRs. Other authors have also reported this type of decrease and have correlated it with a worse prognosis of COVID-19 patients. In the data reported by Marchetti et al. [51], high levels of inflammatory cytokines were observed, however a decrease in coagulation factors was observed. Another study, Andrade et al. [52] also reported a decrease in coagulation factors such as antithrombin as well as a decrease in C-reactive protein. Regarding iron metabolism, factors such as ferritin and transferrin also evaluated in our new detection platform, Lenser and collaborators [53] found patients with a decrease in the levels of these homeostatic factors and correlated it with a negative prognosis of the patients.

One of the limitations of our study is the lack of data on specific APR profiles of other diseases. Although it is true that we have characterized in depth the profile of these proteins in COVID-19 disease, it is a challenge for the future to characterize different diseases with their corresponding profiles. In this work we present a simple, fast and multiparametric methodology, it would also be necessary to translate this novel platform from the bench to the clinical practice. This important step in translational research is one of our next challenges that has begun with this report.

Taking into consideration the results of the work and the characteristics of the disease studied, it can be concluded that a new multiplex platform has been successfully constructed and validated that allows the simultaneous detection and determination of differential profiles of APRs. Furthermore, through this new platform, significant variations have been detected between APRs that can be associated with different types of sepsis and ARDS occurrence in patients with COVID-19 that can be used for patient stratification or therapeutic follow-up.

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Associated Data

In supplementary file, Table S1 and Figure S1 is depicted. Patients' information for both cohorts studied is detailed in a separated spreadsheet Table S2. Complete proteomic data can be requested from the authors of correspondence when necessary.

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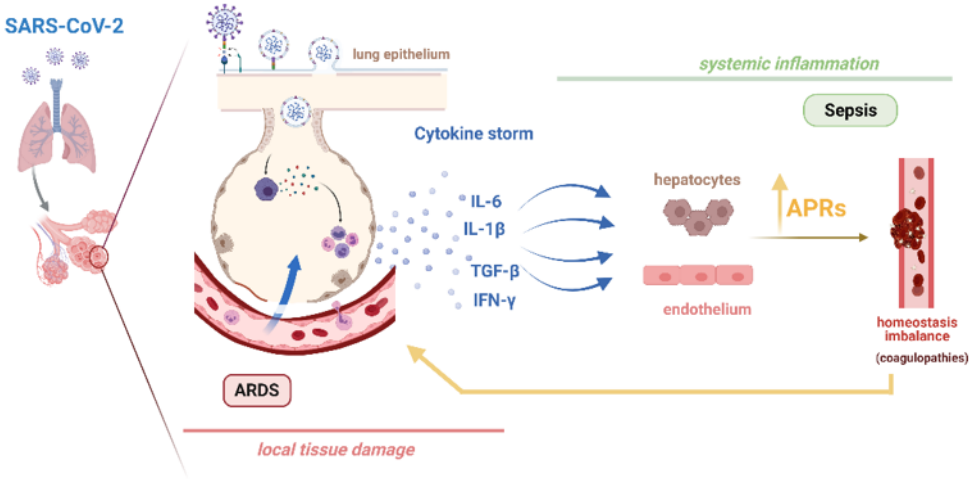

Figure 1. Progression of SARS-CoV-2 infection and development of major disease complications by COVID19, sepsis and ARDS and the role of APRs.

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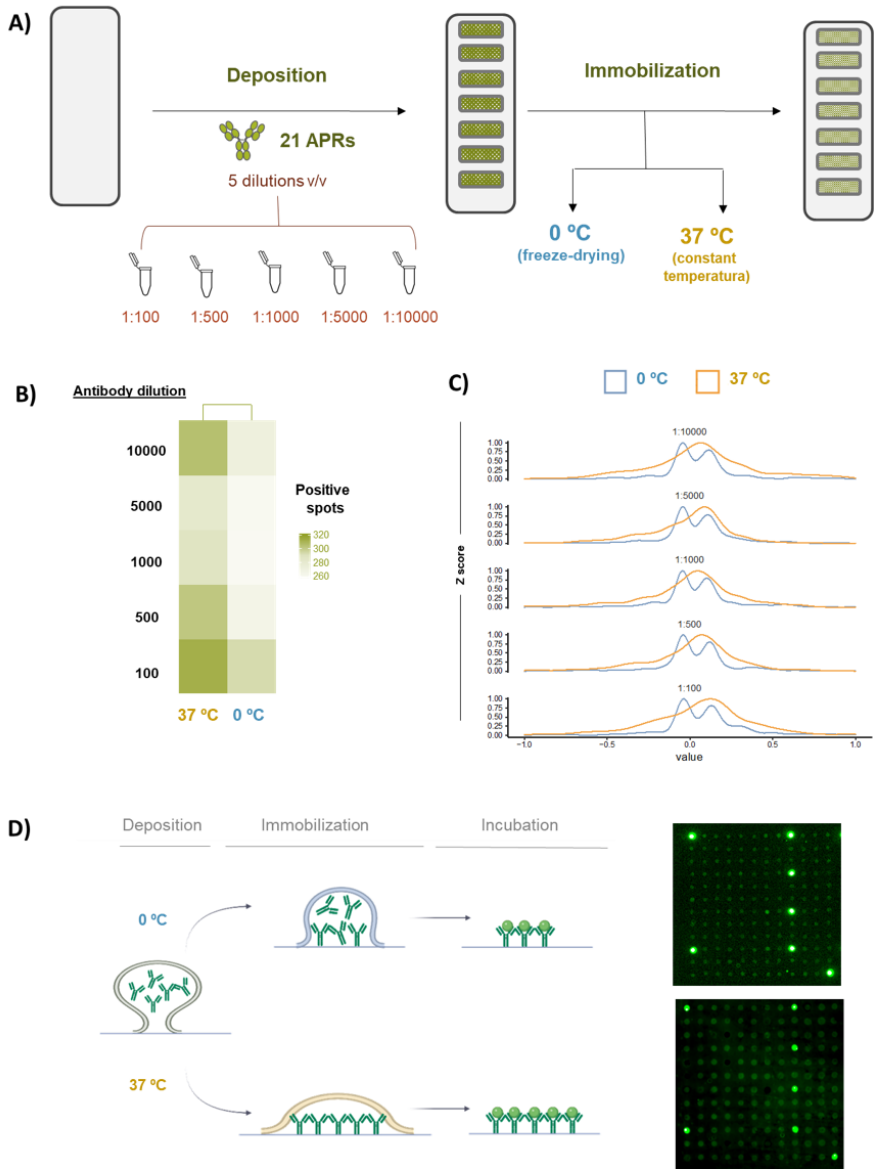

Figure 2 A) Schematic representation of the procedures carried out for the optimization of the APRs microarray. B) Heatmap representing the number of positive spots in each condition studied. C) Descriptive graph of the distribution of the Z-score values of the set of spots of the microarrays. D) Descriptive diagram of the behavior of the spots as a function of the method of immobilization of the antibodies.

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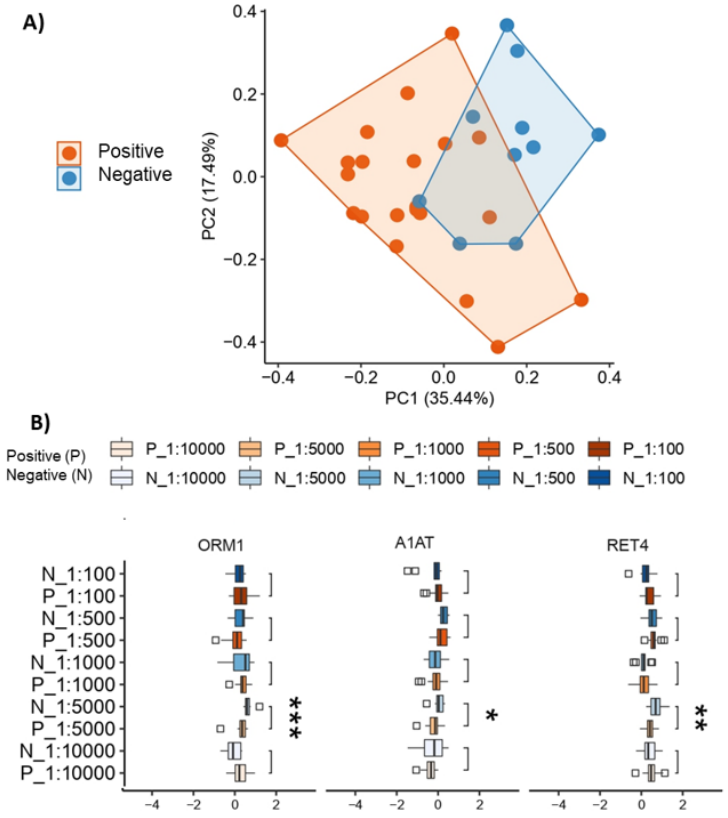

Figure 3 A) PCA with distribution of the different samples studied grouped by COVID-19 positive (orange) and negative (blue). B) Box plots representing the Z-score values obtained for some APRs in negative (blue) and positive (orange) individuals at each of the antibody concentrations deposited on the microarray (The difference between means was evaluated by applying the Wilcoxon rank sum test: p * <0.05, ** <0.01, *** <0.001)

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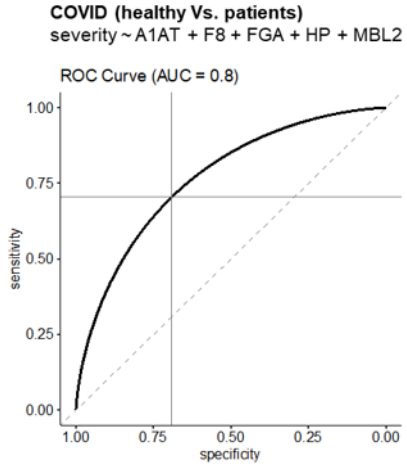

Figure 4 ROC analysis of binomial models of healthy and COVID-19 patients localization data as classification labels (pROC R Package). The initial model evaluated all the proteins with significant differences in the relative abundance (Z ratio) between healthy and patients' profiles (boxplots). Best model was fitted by stepwise Akaike's information criterion (AIC) score analysis (MASS R package)

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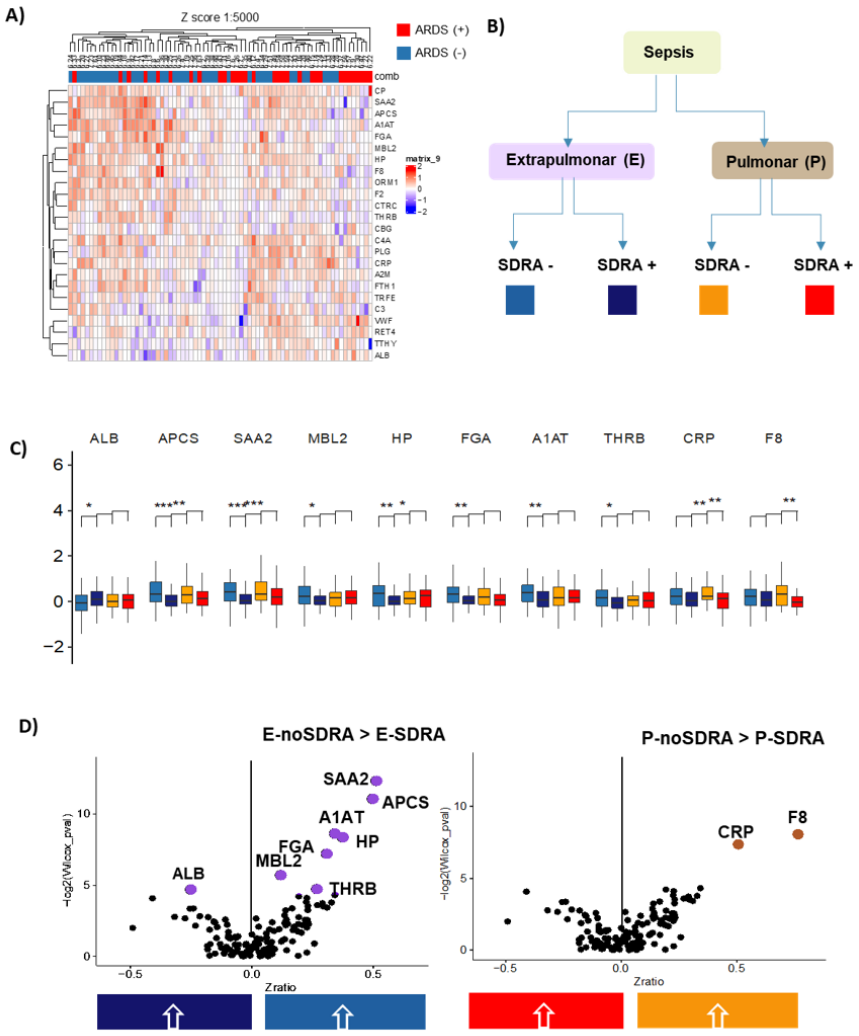

Figure 5 A) Heat map with the Z-score values obtained from the APR microarray at 1:5000 dilution in cohort 2 comparing individuals with ARDS (red) and without the pathology (blue). B) Clinical classification of patient of cohort 2. C) Box plots representing the Z-score values obtained for APRs D) Volcano-plots comparing APRs between individuals who presented extrapulmonary sepsis (E) (left) and pulmonary sepsis (P) (right)

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Table1: APRs and their physiological role. This table collects the information of the functional behavior in inflammation (positive/negative) and the results obtained in sepsis screening.

Protein	Group	Positive/ Negative	Extrapulmonar (E)/ Pulmonar(P)	ARDS
ALB	Albumins	Negative	E	Increase
ORM1	Albumins	Negative	-	-
APCS	Amyloid related	Positive	E	Decrease
RET4	Amyloid related	Negative	-	-
SAA2	Amyloid related	Positive	E	Decrease
TTHY	Amyloid related	Negative	-	-
A2M	Coagulation	Positive	-	-
F2	Coagulation	Positive	-	-
F8	Coagulation	Positive	P	Decrease
FGA	Coagulation	Positive	E	Decrease
PLG	Coagulation	Positive	-	-
THBR	Coagulation	Positive	E	Decrease
VWF	Coagulation	Positive	-	-
C3	Complement	Positive	-	-
C4A	Complement	Positive	-	-
CRP	Complement	Positive	P	Decrease
MBL2	Complement	Positive	E	Decrease
CP	Metal Homesotasis	Positive	-	-
FTH1	Metal Homesotasis	Positive	-	-
HP	Metal Homesotasis	Positive	E	Decrease
TF	Metal Homesotasis	Negative	-	-
A1AT	Serpins	Positive	E	Decrease
CBG	Serpins	Negative	-	-
CTRC	Serpins	Positive	-	-

ALB: albumin; SAA: serum amyloid A; APCS: serum amyloid P; THBR: thrombin; CP: ceruloplasmin; VWF: von Willebrand factor; C3: complement factor C3; C4: complement factor C4; F8: factor VIII; FTH1: ferritin; FGA: fibrinogen; HP: haptaglobin; MBL2: mannose-binding lectin; PGA: plasminogen; CRP: C-reactive protein; RET4: retinol transporter protein; F2: prothrombin; TF: transnferrin; CBG: transcortin; TTHY: transthyretin; CTRC: α-1-antichymotrypsin; A1AT: α-1-antitrypsin; ORM1: α-1-acid glycoprotein; A2M: α-2-macroglobulin.

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