

## Homocysteinylation of alpha 1 antitrypsin as an antigenic target of autoantibodies in seronegative rheumatoid arthritis patients

Tania Colasanti<sup>a,\*</sup>, Danilo Sabatinelli<sup>a</sup>, Carmine Mancone<sup>b</sup>, Alessandra Giorgi<sup>c</sup>, Arbi Pecani<sup>d</sup>, Francesca Romana Spinelli<sup>a</sup>, Alessandra Di Giamberardino<sup>b</sup>, Luca Navarini<sup>e</sup>, Mariangela Speziali<sup>a</sup>, Marta Vomero<sup>a</sup>, Cristiana Barbatì<sup>a</sup>, Carlo Perricone<sup>a</sup>, Fulvia Ceccarelli<sup>a</sup>, Annacarla Finucci<sup>a</sup>, Alessandra Ida Celia<sup>a</sup>, Damiano Currado<sup>e</sup>, Antonella Afeltra<sup>e</sup>, Maria Eugenia Schininà<sup>c</sup>, Vincenzo Barnaba<sup>f,g</sup>, Fabrizio Conti<sup>a</sup>, Guido Valesini<sup>a</sup>, Cristiano Alessandri<sup>a</sup>

<sup>a</sup> Rheumatology Unit, Department of Clinical Internal, Anesthetic and Cardiovascular Sciences, Sapienza University of Rome, Rome, Italy

<sup>b</sup> Department of Molecular Medicine, Proteomics Laboratory, Sapienza University of Rome, Rome, Italy

<sup>c</sup> Department of Biochemical Sciences "A. Rossi Fanelli", Sapienza University of Rome, Rome, Italy

<sup>d</sup> University Hospital "Shefqet Ndroqi", Tirana, Albania

<sup>e</sup> Unit of Allergology, Immunology and Rheumatology, Department of Medicine, Campus Bio-Medico University of Rome, Rome, Italy

<sup>f</sup> Department of Clinical Internal, Anesthetic and Cardiovascular Sciences, Sapienza University of Rome, Rome, Italy

<sup>g</sup> Istituto Pasteur Italia-Fondazione Cenci Bolognietti, Rome, Italy

### ARTICLE INFO

#### Keywords:

Seronegative rheumatoid arthritis  
Rheumatoid arthritis  
Alpha 1 antitrypsin  
Autoantibodies  
Post-translational modifications  
Homocysteine

### ABSTRACT

Rheumatoid arthritis (RA) is a chronic autoimmune disease and rheumatoid factor (RF) and anti-citrullinated protein antibodies (ACPA) are the most frequently detected autoantibodies (autoAbs). To date, more than 20% of RA cases are still defined as seronegative forms (seronegative RA, SN-RA). The aim of this study was to identify new antigenic targets of autoAbs in RA patients, which can also be recognized in SN-RA. Using a proteomic approach, we tested sera from SN-RA patients by analyzing synovial fluid (SF) proteins from these patients. Sera from SN-RA patients revealed a strong reactive spot, corresponding to alpha 1 antitrypsin (A1AT). Reverse-phase nanoliquid chromatography and tandem mass spectrometry (Matrix Assisted Laser Desorption/Ionization-Time Of Flight, MALDI-TOF/TOF) confirmed the presence of A1AT in SF and showed that homocysteinylation was one of the post-translational modifications of A1AT. Homocysteinylation (Hcy)-A1AT immunoprecipitated from SN-RA patients' SFs and *in vitro* modified Hcy-A1AT were used as antigens by Enzyme-Linked Immunosorbent Assay (ELISA) to test the presence of specific autoAbs in sera from 111 SN-RA patients, 132 seropositive (SP)-RA patients, and from 95 patients with psoriatic arthritis, 40 patients with osteoarthritis, and 41 healthy subjects as control populations. We observed that a large portion of SN-RA patients (75.7%), and also most of SP-RA patients' sera (87.1%) displayed anti-Hcy-A1AT autoAbs (anti-HATA). Native A1AT was targeted at a lower rate by SP-RA patients autoAbs, while virtually no SN-RA patients' sera showed the presence of anti-native A1AT autoAbs. In conclusion, anti-HATA can be considered potential biomarkers for RA, also in the SN forms. The discovery of novel autoAbs targeting specific autoantigens can represent higher clinic significance for all RA patients' population.

\* Corresponding author. Rheumatology Unit, Department of Clinical Internal, Anesthetic and Cardiovascular Sciences, Sapienza University of Rome, Viale del Policlinico, 155 -00161, Rome, Italy.

E-mail addresses: [tania.colasanti@gmail.com](mailto:tania.colasanti@gmail.com) (T. Colasanti), [danilo.sabatinelli@gmail.com](mailto:danilo.sabatinelli@gmail.com) (D. Sabatinelli), [carmine.mancone@uniroma1.it](mailto:carmine.mancone@uniroma1.it) (C. Mancone), [alessandra.giorgi@uniroma1.it](mailto:alessandra.giorgi@uniroma1.it) (A. Giorgi), [arbipecani@gmail.com](mailto:arbipecani@gmail.com) (A. Pecani), [francescaromana.spinelli@uniroma1.it](mailto:francescaromana.spinelli@uniroma1.it) (F.R. Spinelli), [alessandra.digiamberardino@uniroma1.it](mailto:alessandra.digiamberardino@uniroma1.it) (A. Di Giamberardino), [l.navarini@unicampus.it](mailto:l.navarini@unicampus.it) (L. Navarini), [mariangela.speziali@uniroma1.it](mailto:mariangela.speziali@uniroma1.it) (M. Speziali), [marta.vomero@gmail.com](mailto:marta.vomero@gmail.com) (M. Vomero), [cristiana.barbati1@gmail.com](mailto:cristiana.barbati1@gmail.com) (C. Barbatì), [carlo.perricone@gmail.com](mailto:carlo.perricone@gmail.com) (C. Perricone), [fulvia.ceccarelli@uniroma1.it](mailto:fulvia.ceccarelli@uniroma1.it) (F. Ceccarelli), [finini@hotmail.it](mailto:finini@hotmail.it) (A. Finucci), [celiaalessandraida@gmail.com](mailto:celiaalessandraida@gmail.com) (A.I. Celia), [d.currado@unicampus.it](mailto:d.currado@unicampus.it) (D. Currado), [a.afeltra@unicampus.it](mailto:a.afeltra@unicampus.it) (A. Afeltra), [eugenia.schinina@uniroma1.it](mailto:eugenia.schinina@uniroma1.it) (M.E. Schininà), [vincenzo.barnaba@uniroma1.it](mailto:vincenzo.barnaba@uniroma1.it) (V. Barnaba), [fabrizio.conti@uniroma1.it](mailto:fabrizio.conti@uniroma1.it) (F. Conti), [guido.valesini@uniroma1.it](mailto:guido.valesini@uniroma1.it) (G. Valesini), [cristiano.alessandri@uniroma1.it](mailto:cristiano.alessandri@uniroma1.it) (C. Alessandri).

<https://doi.org/10.1016/j.jaut.2020.102470>

Received 11 December 2019; Received in revised form 18 April 2020; Accepted 19 April 2020

0896-8411/ © 2020 Elsevier Ltd. All rights reserved.

## 1. Introduction

Rheumatoid arthritis (RA) is an inflammatory autoimmune disease that causes pain, swelling and stiffness in the affected joints. The current classification criteria for RA include two autoantibodies (autoAbs), that are rheumatoid factor (RF) and anti-citrullinated protein antibodies (ACPA) [1]. Several studies have reported the presence of these autoAbs in sera of subjects that eventually develop RA up to 10 years before the onset of the clinical manifestations [2], emphasizing their key role in the diagnosis of the disease. The recent recognition of various autoAbs against post-translationally modified proteins opened new perspectives to diagnosing RA and predicting the course of the disease [3]. Nevertheless, it is possible that some patients display clinical manifestations suggestive of RA, but they are persistently negative for the conventional RA immunological tests. These are referred as “seronegative RA” (SN-RA) patients.

The concept of SN-RA arose in 1978 from the observation of a number of patients with the classical clinical manifestations, but persistently negative for the RF. SN-RA patients may develop ACPA and RF in the serum, evolving into a seropositive RA, or remain SN. The lack of evidence of circulating autoAbs in these cases may represent a real absence or can be the consequence of the limits of the currently available methods for the detection of new antigenic targets/new Ab specificities. In this context, the discovery of novel autoAbs targeting a specific molecule may be useful and have higher clinic significance for RA patients.

By a molecular point of view, many processes are involved in RA pathogenesis. As above mentioned, post-translational modifications (PTMs) can occur in the disease and play a leading role in favouring an autoimmune response; particularly, citrullination and carbamylation have been extensively studied and immunologically characterized [4,5]. Recently, other autoAbs recognizing post-translationally modified proteins have also gained attention. They include autoAbs directed to fragmented immunoglobulin (anti-hinge Abs), proteins modified by molecules formed under oxidative stress [6], and autoAbs recognizing acetylated proteins [7]. Therefore, it can be assumed that PTMs play an important role in inducing autoimmunity and, specifically, many of them are widely described in rheumatic autoimmune diseases [8], in which they can result from the action of environmental factors (i.e., smoke, air pollution) [9], in genetically predisposed subjects (i.e., HLA shared epitope) [10–12]. In this regard, Padyukov et al. [13,14] performed a genome-wide association study in ACPA-positive and ACPA-negative RA patients. More than 1.7 million single nucleotide polymorphisms were studied for association with the disease. The comparison between the two groups displayed significant risk allele frequency differences, which were mainly confined to the HLA region. These data support the idea that genetic backgrounds contribute differently to the two RA disease subsets characterized by ACPA status [13,14].

In the light of all these considerations, the aim of the present study was to identify new possible specific antigenic target(s) of autoAbs in RA, as well as the immunological characterization, with a particular focus on SN-RA patients. For this purpose, we used an autoAb-based screening method, a proteomic approach using synovial fluid (SF) that represents a precise, well-established, and at the vanguard tool for the detection of target antigens.

Reverse-phase nanoliquid chromatography (nanoLC) and tandem mass spectrometry (Matrix Assisted Laser Desorption/Ionization-Time Of Flight, MALDI-TOF/TOF) have allowed discovering some PTMs of the protein(s) in SF. Finally, we analyzed the presence of specific serum autoAbs using Enzyme-Linked ImmunoSorbent Assay (ELISA).

## 2. Material and methods

### 2.1. Patients, serum and SF sample preparation

For this study consecutive patients, attending the Arthritis Center of Sapienza University of Rome and the Unit of Allergology, Immunology and Rheumatology, Campus Bio-Medico University of Rome, were enrolled.

They presented clinical features that were consistent with a diagnosis of RA, as stated in the 2010 American College of Rheumatology (ACR) classification criteria [1]. SN-RA cases were defined as persistently negative (at least 2 times) for conventional RA tests (RF and ACPA). Both seropositive (SP)- and SN-RA patients' sera have also been previously tested for the presence of anti-fetal calf serum carbamylated proteins (anti-FCS CarP) Abs by ELISA [15].

Consecutive patients with psoriatic arthritis (PsA), with osteoarthritis (OA), and age- and sex-matched healthy subjects (HS) were also included in the study.

After obtaining informed consent from each patient, in accordance with the Declaration of Helsinki, a venous blood draw was performed. After collection, the whole blood was allowed to clot, by leaving it at room temperature for 15–30 min. Then, the clot was removed by centrifuging at  $2000 \times g$  for 10 min in a refrigerated centrifuge. The serum samples obtained were aseptically aliquoted and stored at  $-80^\circ\text{C}$  until the assay.

SFs were obtained during joint aspiration from consecutive SN-RA patients with swollen and tender joints, attending the Arthritis Center of Sapienza University of Rome. SF samples were centrifuged at  $1000 \times g$  for 15 min to remove cells and cellular debris [16]. The supernatants were aseptically removed and stored at  $-80^\circ\text{C}$  in aliquots until the subsequent analyses.

All experiments involving patients were approved by the local Ethical Committee (Prot. N. 5460).

### 2.2. Digestion of hyaluronic acid and depletion of abundant proteins in SF samples

In order to facilitate the handling of SF, we used a highly active hyaluronidase (HSE) from *S. hyalurolyticus* (Sigma Aldrich, USA) to digest hyaluronic acid and thereby reduce viscosity.

Following the protocol developed by Liao and colleagues [17], only microgram quantities of the enzyme were needed to treat 1 ml of SF, so very small amounts of exogenous protein were introduced during digestion.

HSE buffer (60 mM NaOAc, 1 mM EDTA [pH 6.0]) was added to 1 vial of HSE to a final concentration of 1300 units/ml, to prepare enzyme stock solution. Hyaluronic acid digestion was carried out by mixing 1 ml of SF with 40  $\mu\text{l}$  of 25x HSE buffer and 100  $\mu\text{l}$  of the HSE stock solution. The reaction mixture was incubated at  $37^\circ\text{C}$  for 4 h, after which significant reduction in sample viscosity was observed. HSE-treated SFs were centrifuged at  $14,000 \times g$  for 15 min to pellet insoluble material [17].

Human albumin (HA) is the most abundant protein in SF. Although IgG levels in normal SF are low, in patients with RA their levels are similar to those of serum, with an average of 9.5 mg/ml. In order to remove HA and IgG, HSE-treated SFs were subjected to affinity depletion with Aurum Serum Protein Mini kit (BioRad Laboratories, USA). This process was estimated to enrich the remaining SF components by effectively removing up to 90% of the abundant proteins. These two proteins can mask the presence of many co-migrating proteins, as well as limit the amount of total protein that can be resolved through 2 dimensional electrophoresis (2DE) or other techniques. For these reasons, this kit was used to maximize resolution of proteins of interest in SF, improving and supporting protein discovery and identification, and optimizing the subsequent SF analysis by monodimensional and 2DE [18].

### 2.3. Analysis of SF samples

A pool from 5 SN-RA patients' SFs, digested with HSE and HA/IgG depleted (hereafter simply referred as SFs), was loaded into 2DE. Isoelectrofocusing (IEF) was firstly performed on 7-cm ReadyStrip immobilized pH gradient (IPG) Strips with a 3–10 pH range (BioRad Laboratories, USA), by the use of the Protean IEF Cell Apparatus (BioRad Laboratories, USA), and then performed on 7-cm ReadyStrip IPG Strips with a 5–8 pH range (BioRad Laboratories, USA), to better resolve this specific pH region. The second dimension was performed on a 10% Sodium Dodecyl Sulphate-PolyAcrylamide Gel Electrophoresis (SDS-PAGE), after equilibrating the strips for 10 min in SDS Equilibration buffer, containing 50 mM Tris HCl pH 8.8, 6 M urea, 30% glycerol, 2% SDS, 2% dithiothreitol (DTT), and 2.5% iodoacetamide (IAA). Gels were then stained by colloidal Coomassie blue (Sigma Aldrich, USA) or used for Western blot analysis alternatively, incubating with a pool from 5 SN-RA patients' sera diluted 1:50. Horseradish peroxidase (HRP)-conjugate goat anti-human IgG (BioRad Laboratories, USA) were used as second Abs, and the reactions were developed using 3-3' diaminobenzidine (Sigma Aldrich, USA) as a substrate, in the presence of H<sub>2</sub>O<sub>2</sub> (Roche Diagnostics, Switzerland).

After the staining, selected blue-stained spots were excised from gel and subjected to tryptic proteolysis. Briefly, after some wash steps in 50 mM ammonium bicarbonate and 50% acetonitrile (ACN) in 50 mM ammonium bicarbonate to destain the spots, and a step to dry them with absolute ACN, 100 ng of trypsin in 25 mM ammonium bicarbonate were added, and let to incubate overnight at 37 °C. MALDI-TOF mass spectrometry (MS) analyses of peptide tryptic mixtures were performed by an AutoFlex II instrument (Bruker Daltonics, USA), equipped with a 337 nm nitrogen laser and operating in reflector mode. Identification by peptide mass fingerprint (PMF) was performed, using the Mascot search engine (version 2.6.2) against human SwissProt database [(SwissProt 2019\_02 (559228 sequences; 200905869 residues)]. Up to two missed cleavage, 50 ppm measurement tolerance, oxidation at methionine (variable modification) and carbamidomethylation at cysteine (fixed modification) were considered. Identifications were validated when the probability-based MOlecular Weight SEarch (MOWSE) protein score was significant, according to Mascot.

Reverse-phase nanoLC-MALDI-TOF/TOF were used to analyze SF, not only in order to identify the proteins, but also to discover proteins PTMs.

Five µg of proteins from SF were treated with 10 mM DL-DTT and incubated for 30 min at 56 °C for cysteine reduction, then the cysteines were alkylated with 55 mM IAA (20 min at room temperature, in the dark). The proteins in the sample were precipitated overnight at –20 °C, using four volumes of 100% acetone, and subsequently resuspended in 30 µl of a solution containing 50 mM ammonium bicarbonate and 2 M urea. Protein mixture was digested overnight at 37 °C with 0.2 µg of trypsin. The peptide mixture obtained was desalted and filtered through a C18 microcolumn ZipTip (Merck Millipore, USA), and eluted from the C18 bed, using 10 µL of 80% ACN/0.1% trifluoroacetic acid (TFA). The organic component was once again removed by evaporation in a vacuum centrifuge and peptides were resuspended in a suitable nanoLC injection volume (typically 3–10 µl) of 2.5% ACN/0.1% TFA. A more detailed description of this protocol is given in Supplementary File 1, following the procedure described by Del Ben and colleagues [19].

### 2.4. Isolation of homocysteinylated alpha 1 antitrypsin (Hcy-A1AT) from SF

Homocysteinylated (Hcy) proteins were immunoprecipitated by affinity chromatography from a pool of 5 SN-RA patients' SFs, using rabbit IgG anti-Hcy proteins (5 µg; Abcam, UK). Subsequently, immunoprecipitated Hcy proteins were used to isolate modified A1AT, using goat IgG anti-A1AT (5 µg; Abcam, UK). In every reaction,

irrelevant IgG were used as a negative control (5 µg). In brief, following manufacturer's instructions for Thermo Scientific Pierce Direct IP Kit (ThermoFisher Scientific, USA), the SF pool was mixed with Control Agarose Resin slurry and stirred in a rotary shaker for 1 h at 4 °C, to preclear nonspecific binding. After centrifugation (1000 × g for 1 min), proteins of interest were immunoprecipitated from the precleared samples, as indicated.

The immunoprecipitated (IP) samples underwent buffer exchange and concentration by Amicon Ultra-0.5 Centrifugal Filter Devices (Merck Millipore, USA) and then used for the subsequent analyses.

Protein content was determined by the Bradford assay (BioRad Laboratories, USA).

### 2.5. Protein modification

For *in vitro* modification of A1AT, the protocol described by Perla et al. [20] and Perla-Kajan et al. [21] was carried out. Briefly, native A1AT (Abcam, UK) was dissolved at 1 mg/ml in 0.1 M phosphate-buffered saline (PBS) pH 7.4, 0.2 mM EDTA, 0.9 M NaCl, and modified by incubating with 50 mM L-Hcy-thiolactone-HCl (Sigma Aldrich, USA) for 16 h at 37 °C [22,23]. The protein sample underwent buffer exchange and concentration by Amicon Ultra-0.5 Centrifugal Filter Devices (Merck Millipore, USA) and then used for the subsequent analyses. Protein content was determined by the Bradford assay (BioRad Laboratories, USA).

### 2.6. SDS-PAGE and Western blot of protein samples

In order to check HA/IgG depletion, SF samples containing 30 µg of proteins were separated onto a 10% SDS-PAGE in denaturing conditions, before and after the depletion protocol. Gel was stained with colloidal Coomassie blue (Sigma Aldrich, USA).

Protein samples (native A1AT, *in vitro* modified Hcy-A1AT, IP Hcy-A1AT, 10 µg each) and FCS CarP (10 µg), obtained following the protocol described by Shi et al. [4] and used as a control to exclude Ab cross-reactivity, were loaded onto a 10% SDS-PAGE in denaturing conditions. After electrophoresis, the proteins were transferred onto polyvinylidene difluoride membrane (Amersham Hybond-ECL, GE Healthcare Europe, Austria) by means of a Trans-Blot transfer cell (BioRad Laboratories, USA). For the detection of protein PTMs, to improve their retention, the method described by Colasanti et al. was used [24]. The membranes were then blocked in 5% non-fat dry milk (Euroclone, Italy) in Tris-buffered saline containing 0.1% Tween 20 (TBS-T) for 1 h at room temperature, rinsed, and incubated with rabbit IgG anti-A1AT, anti-Hcy proteins (Abcam, UK), and anti-carbamyl-lysine (Cell Biolabs, Inc., USA) as primary Abs, all used at a dilution of 1:1000 in TBS-T containing 5% non-fat dry milk (Euroclone, Italy). Excess primary Ab was removed by washing the membranes in TBS-T. The membranes were then incubated with goat anti-rabbit IgG HRP-conjugate (BioRad Laboratories, USA), diluted 1:3000 in TBS-T with 5% non-fat dry milk (Euroclone, Italy). The reaction was developed using the chemiluminescent HRP detection reagent Luminata Forte (Merck Millipore, USA).

### 2.7. ELISA to detect serum Abs

Polystyrene 96-well plates (Maxisorp, Nunc, USA) were coated and incubated overnight at 4 °C with 100 µl/well of (IP or *in vitro* modified) Hcy-A1AT in 0.05 M Na<sub>2</sub>CO<sub>3</sub>/NaHCO<sub>3</sub> buffer, pH 9.6. Coated plates were then washed 4 times with PBS containing 0.05% Tween 20 (PBS-T). Plates were blocked for 1 h at 37 °C with 3% non-fat dry milk (Euroclone, Italy) in PBS-T. After 4 washings, the wells were incubated for 1 h at 25 °C with patients' sera diluted 1:50 in PBS-T containing 1% non-fat dry milk (Euroclone, Italy). Rabbit polyclonal specific anti-Hcy proteins and anti-A1AT Abs (Abcam, UK) were used as a positive control. After 4 washes with PBS-T, the plates were incubated with goat

anti-human or anti-rabbit IgG HRP-conjugate (BioRad Laboratories, USA), diluted 1:3000 in 1% non-fat dry milk (Euroclone, Italy) in PBS-T for 1 h at 25 °C. The plates were washed 3 times, the bound peroxidase was revealed incubating with O-phenylenediamine dihydrochloride (Sigma Aldrich, USA) for 20 min, and color development was stopped with 2 M H<sub>2</sub>SO<sub>4</sub>. Absorbance was measured at 490 nm in a spectrophotometer (iMark Microplate Reader, BioRad Laboratories, USA). Data were presented as the mean optical density (OD) corrected for background (wells without coated antigen). Forty-one HS sera were tested and a cut-off value was established at a mean of OD  $\pm$  3 standard deviations (SDs) of HS sera.

The intra-assay coefficient of variation (CV) was 2.3% and the inter-assay CV was 4.3%.

Parallel experiments were performed using native A1AT (Abcam, UK) as an antigen. The above-described protocol was employed, with slight modifications: patients' sera were diluted 1:100 in 1% non-fat dry milk in PBS-T and color development was stopped with 2 M H<sub>2</sub>SO<sub>4</sub> after an incubation time of 5 min with O-phenylenediamine dihydrochloride to reveal the bound peroxidase.

The intra-assay CV ranged from 2.9 to 4.6% and the inter-assay CV ranged from 4.7 to 6.4%.

## 2.8. Purification of human Abs

The *in vitro* modified Hcy-A1AT (100 µg) was coupled to Sepharose 4B (Sigma Aldrich, USA), according to the manufacturer's instructions. A pool of sera from 5 SN-RA patients positive for anti-Hcy-A1AT Abs (hereafter referred as anti-HATA) was diluted in PBS and applied to the Sepharose-galactose column. Bound Abs were eluted with 0.1 M glycine pH 2.5, and dialyzed against PBS. The concentration of the Ab sample was determined by the Bradford assay (BioRad Laboratories, USA). After purification, affinity-purified anti-HATA were tested by ELISA to exclude variation in binding reactivity, and for the inhibition assay.

## 2.9. Inhibition assay to test binding specificity

To evaluate the antigen-Ab binding specificity, an inhibition assay was performed, using the protocol described by Colasanti et al. [25]. Briefly, human purified anti-HATA (1 µg) were incubated overnight in a polystyrene 96-well plate (Maxisorp, Nunc, USA) with different concentrations (0.5–2 µg) of *in vitro* modified Hcy-A1AT and of IP Hcy-A1AT, for a total reaction volume of 100 µl each in PBS.

The inhibition assay was also employed to test binding specificity of rabbit polyclonal anti-Hcy protein Ab. In order to this, the intended Ab was incubated overnight with *in vitro* modified Hcy-A1AT, IP Hcy-A1AT and FCS CarP, following the above-described protocol.

## 2.10. Statistical analysis

Data were statistically analyzed using GraphPad Prism (Version 6 GraphPad Software, CA, USA). Since values were not normally distributed according to Kolmogorov-Smirnov, non-parametric statistics (median and percentiles) have been used. The Spearman rank test was used to analyze the correlation between autoAb levels and disease activity score (DAS) 28, erythrocyte sedimentation rate (ESR) and C reactive protein (CRP). The Mann Whitney test was used to investigate the differences between two groups. Receiver operating characteristic (ROC) analyses were used to evaluate the discriminatory power of the assays. P values < 0.05 were considered as significant.

## 3. Results

### 3.1. Demographic, clinical, serological and therapeutic features of RA patients

The characteristics of the patients included in this group are

**Table 1**

Demographic, clinical, serological and therapeutic features of SN-RA patients (n = 111).

Characteristics	Value
<b>Demographic parameters</b>	
Sex, female/male	88/23
Age, median (25°–75° percentile), years	55 (46.25–66.75)
Disease duration, median (25°–75° percentile), months	72 (24–132)
<b>Clinical and serological parameters</b>	
DAS28 <sub>ESR</sub> , median (25°–75° percentile)	2.95 (2.1–3.76)
ESR, median (25°–75° percentile), mm/h	23 (10–45.25)
CRP, median (25°–75° percentile), mg/dl	0.4 (0.185–1)
Anti-FCS CarP Abs positive, n. (%)	24 (21.6)
<b>Concomitant treatment(s)</b>	
Methotrexate, n. (%)	51 (45.9)
Targeted synthetic DMARDs, n. (%)	31 (27.9)
Biologic DMARDs, n. (%)	29 (26.1)
Glucocorticoids, n. (%)	34 (30.6)

DAS, Disease Activity Score; ESR, Erythrocyte Sedimentation Rate; CRP, C Reactive Protein; Anti-FCS CarP Abs, Anti-Fetal Calf Serum Carbamylated Proteins Antibodies; DMARDs, Disease Modifying Anti-Rheumatic Drugs.

**Table 2**

Demographic, clinical, serological and therapeutic features of SP-RA patients (n = 132).

Characteristics	Value
<b>Demographic parameters</b>	
Sex, female/male	99/33
Age, median (25°–75° percentile), years	59.5 (53–70.5)
Disease duration, median (25°–75° percentile), months	48 (24–120)
<b>Clinical and serological parameters</b>	
DAS28 <sub>ESR</sub> , median (25°–75° percentile)	3.25 (1.9–4.15)
ESR, median (25°–75° percentile), mm/h	39 (17.75–71.25)
CRP, median (25°–75° percentile), mg/dl	0.5 (0.2–1.3)
RF positive, n. (%)	121 (91.7)
ACPA positive, n. (%)	99 (75)
Anti-FCS CarP Abs positive, n. (%)	80 (60.6)
<b>Concomitant treatment(s)</b>	
Methotrexate, n. (%)	66 (50)
Targeted synthetic DMARDs, n. (%)	26 (19.7)
Biologic DMARDs, n. (%)	40 (30.3)
Glucocorticoids, n. (%)	61 (46.2)

DAS, Disease Activity Score; ESR, Erythrocyte Sedimentation Rate; CRP, C Reactive Protein; Anti-FCS CarP Abs, Anti-Fetal Calf Serum Carbamylated Proteins Antibodies; DMARDs, Disease Modifying Anti-Rheumatic Drugs.

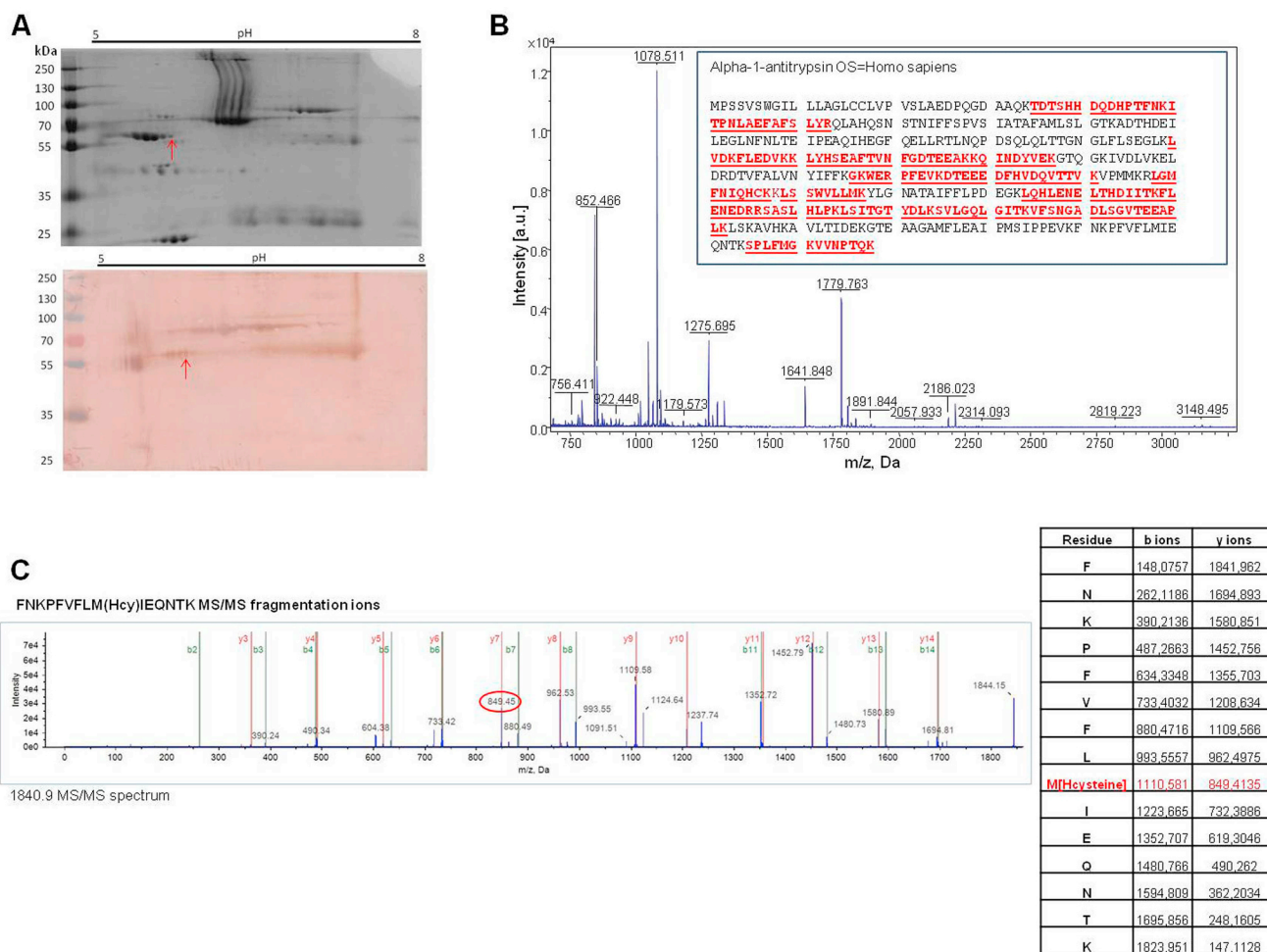
summarized in [Tables 1 and 2](#), divided into SN- and SP-RA patients, respectively. All the patients were previously screened for conventional serological tests (ACPA and RF) and for the presence of anti-FCS CarP Abs. SN-RA patients' sera were found to be negative for both ACPA and RF. For this study, no RA patients with skin involvement were enrolled.

### 3.2. Identification of Hcy-A1AT as an antigenic target in SF of SN-RA patients

In order to identify a possible antigenic target, SF from SN-RA patients were initially digested with HSE, depleted of HA/IgG and protein content separated by SDS-PAGE, to assess the protocol successfully ([Fig. S1](#)).

At first, SF protein separation by 2DE on strip with 3–10 pH range revealed several spots, with molecular weights approximately between 40 and 70 kDa, most of all in the 5–8 pH region ([Fig. S2](#)). Consequently, SF protein separation was optimized, by using IPG strips focused in 5–8 pH range. After IEF, a 2DE gel was run and stained ([Fig. 1A](#), upper panel), and simultaneously another gel was transferred onto nitrocellulose membrane, and analyzed with a pool of sera from 5 SN-RA patients. The Western blot analysis detected an antigen more strongly recognized by SN-RA patients' serum Abs, as shown in [Fig. 1A](#) (lower





**Fig. 1.** Identification of homocysteinylated (Hcy)-alpha 1 antitrypsin (A1AT) as an antigenic target in synovial fluid (SF) of seronegative (SN)-RA patients. (A) Two-dimensional electrophoresis (top) and immunoblotting (bottom) of a pool of SFs from 5 SN-RA patients using a pool of sera from 5 SN-RA patients. The spot (indicated by the red arrow) was excised and analyzed by mass spectrometry (MS). (B) Peptide mass fingerprint (PMF) related to A1AT. Covered sequence is indicated in the box, in red boldface and underscored. (C) MS/MS spectrum (left) and ion fragmentation pattern (right) of 1840.9 *m/z*. Y ion containing the homocysteine modification is red circled in the spectrum (left) and indicated in red in the table (right).

panel). The PMF indicated the antigen as A1AT (Fig. 1B).

Reverse-phase nanoLC-MALDI-TOF/TOF MS analysis confirmed the presence of A1AT in SN-RA SF and revealed homocysteinylolation as one of the PTMs of this protein (Fig. 1C and Supplementary File 2).

### 3.3. Protein samples and specificity of Abs targeting PTMs

SFs were tested for the presence of Hcy-A1AT also by immunoprecipitation, and used to isolate Hcy-A1AT, first using polyclonal anti-Hcy proteins and then anti-A1AT Abs. In order to verify a possible Ab cross-reactivity with other PTMs, anti-carbamyl-lysine Abs and FCS CarP were also used. The IP Hcy-A1AT, the FCS CarP, the *in vitro* modified Hcy-A1AT (all purified by buffer exchange and concentrated) and native A1AT were analyzed by Western blot, using anti-Hcy proteins, anti-carbamyl-lysine and anti-A1AT Abs. As shown in Fig. 2A–C, no Ab cross-reactivity occurred.

By ELISA, we tested anti-Hcy proteins Ab previously blocked with *in vitro* modified Hcy-A1AT, IP Hcy-A1AT and FCS CarP, respectively (Fig. 2D). No change in reactivity resulted when the Ab was pre-incubated with FCS CarP. On the contrary, the Ab reactivity was gradually reduced by the incubation with both Hcy proteins, in a dose-dependent manner (Fig. 2D).

All together, these experiments confirmed that the protocols for protein isolation were correctly performed and that no Ab cross-

reactivity occurred, concluding that Abs were specific for the intended PTMs.

### 3.4. Detection of Abs directed to Hcy-A1AT and native A1AT

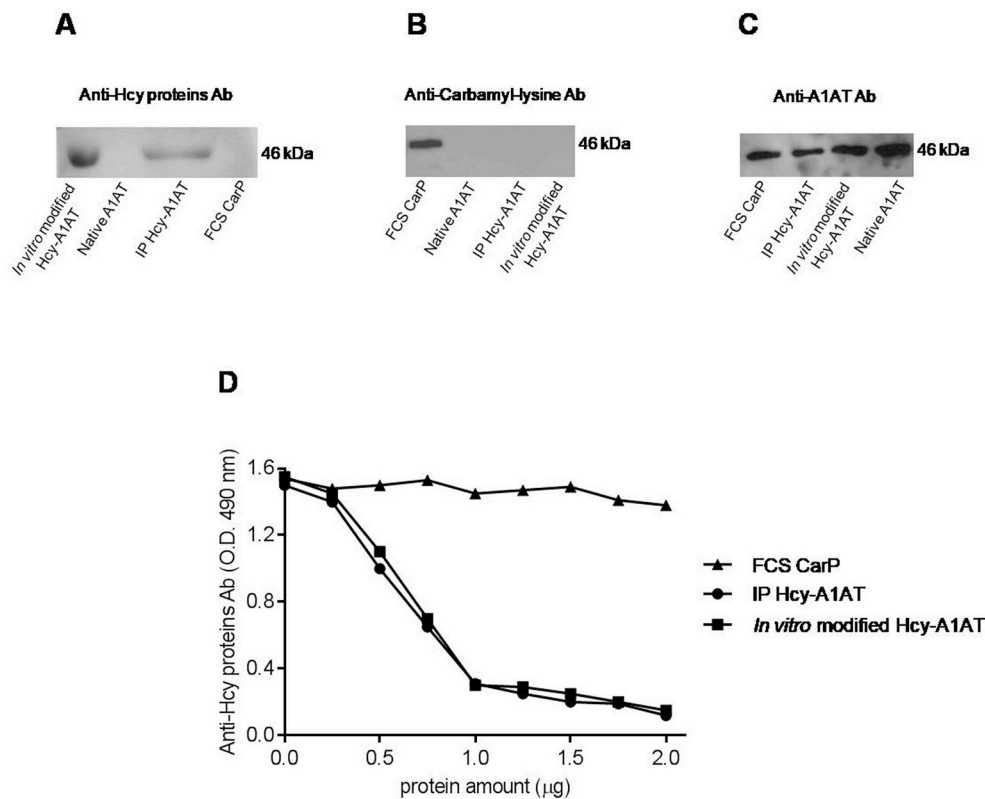
ELISAs were carried out using as antigens both the *in vitro* modified and the IP Hcy-A1AT, and the results were virtually the same.

The analysis of the sera by ELISA showed that 84/111 (75.7%) SN-RA patients, 115/132 (87.1%) SP-RA (RA) patients, 24/95 (25.3%) patients with PsA, 6/40 (15%) patients with OA, and 0/41 HS displayed anti-HATA (Fig. 3B). The occurrence of anti-HATA IgG was significantly greater in patients with RA, SN-RA, PsA and OA compared with HS ( $P < 0.0001$  for all the groups *versus* HS). Moreover, the occurrence in all RA (SN and SP) patients was also significantly greater with respect to PsA and OA patients ( $P < 0.0001$ , respectively). ODs of anti-HATA IgG are reported in Fig. 3B. No significant correlation was found between anti-HATA and clinical or serological parameters in SN- or SP-RA patients.

In the cohort of SN-RA, the percentage of patients positive for anti-HATA was higher than the percentage of patients positive for anti-FCS CarP Abs, previously tested and reported in Table 1.

Of note, 54.5% (72/132) of SP- and only 1.8% (2/111) of SN-RA patients showed anti-native A1AT Abs (Fig. 3A).

ROC analysis showed the statistically significant differences



**Fig. 2.** Testing of specificity of protein and antibody samples. Anti-Hcy proteins (A), anti-Carbamyl-lysine (B) and anti-A1AT (C) antibodies (Abs) were incubated with immunoprecipitated (IP) Hcy-A1AT, Fetal Calf Serum carbamylated proteins (FCS CarP), *in vitro* modified Hcy-A1AT and native A1AT, by Western blot. The images show the *in vitro* modification and immunoprecipitation protocols had been correctly performed. The Abs used had shown proper functioning and specificity, confirming the isolation of Hcy-A1AT. (D) The specificity of the anti-Hcy proteins Ab binding to its molecular target was also showed by ELISA, in which no changes in reactivity were observed assaying the anti-Hcy proteins Ab previously blocked with FCS CarP, while the binding to IP and the *in vitro* modified Hcy-A1AT was able to abolish the anti-Hcy proteins Ab reactivity.

between SN-RA patients and HS (Area under curve, AUC = 0.9204, Fig. 4A) and between the whole RA patients' population and HS (AUC = 0.9517, Fig. 4B) for the presence of anti-HATA. The ROC curves for anti-HATA, discriminating between SN-RA patients and disease controls (AUC = 0.6539) and between the whole RA population and disease controls (AUC = 0.819), are reported in Fig. 4C and D.

The pre-absorption of the human affinity-purified anti-HATA with Hcy-A1AT protein preparations completely inhibited the Ab reactivity, confirming the specificity of the ELISA (data not shown).

These findings strongly suggest a role for Hcy-A1AT as an antigenic target for autoAbs in RA patients' sera, including SN-RA patients.

#### 4. Discussion

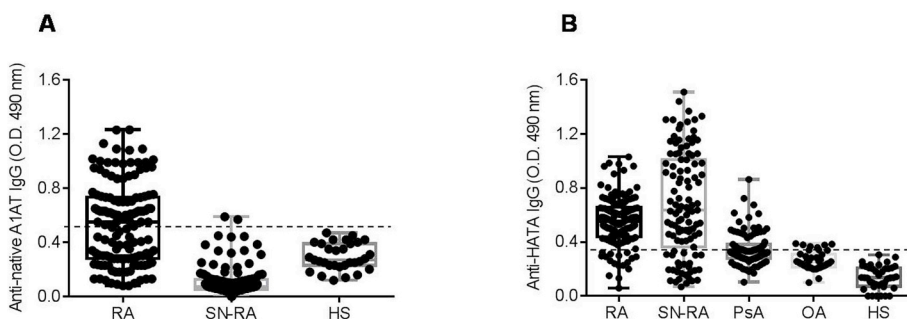
Identifying potential specific antigenic targets in RA patients is considered as a particular challenge, especially in the SN-RA forms, which represent about 20% of the total RA population. Indeed, in SN-RA subjects a definite diagnosis of RA is sometime difficult. Generally, the lack of molecular targets of Abs is actually due to limited methodological approaches, rather than a real absence. In order to this, we

used an autoantibody-based screening method (immunoproteomic and MS) for the recognition of target proteins in SF of SN-RA patients, combined with the SF analysis (reverse-phase nanoLC-MALDI-TOF/TOF) for the identification of proteins and their PTMs.

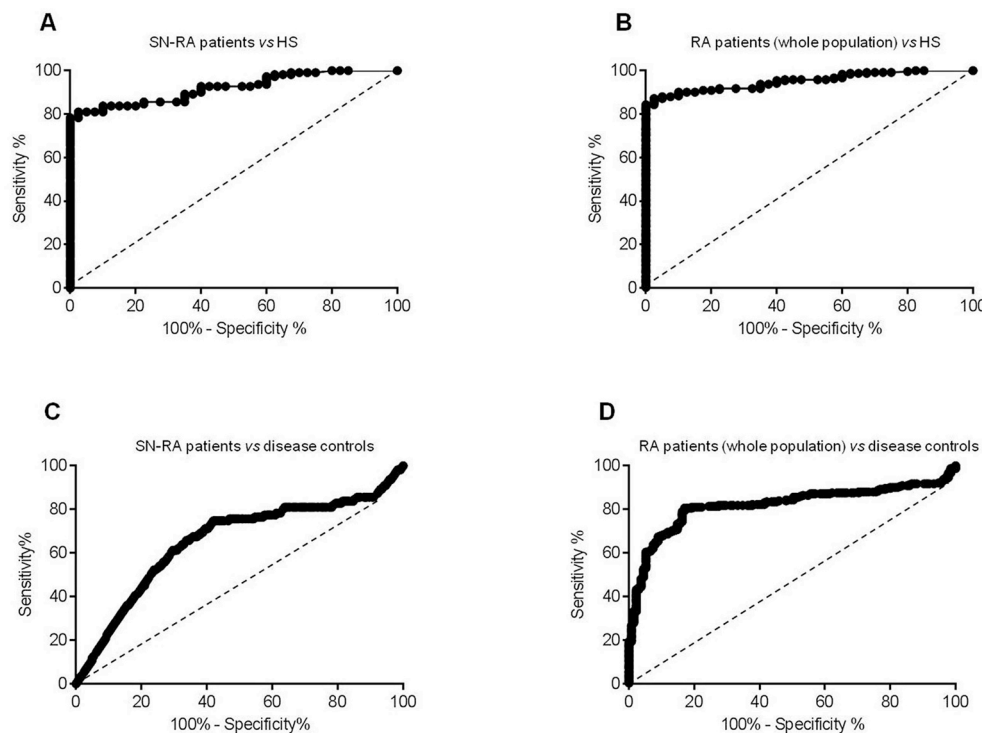
In order to identify specific biomarkers for SN-RA, we firstly carried out a 2DE-MS analysis, which revealed the presence of A1AT in SF of these patients. However, ELISA didn't point out the presence of Abs recognizing the native A1AT in SN-RA patients' sera. Consequently, we performed the SF analysis by reverse-phase nanoLC-MALDI-TOF/TOF MS, which allowed us to identify the presence of some A1AT PTMs. ELISA showed Hcy-A1AT as an antigenic target of autoAbs in sera from SN-RA patients and in whole RA patients' population.

We also showed the specificity of the anti-Hcy proteins Ab, since no reactivity toward FCS CarP was revealed by Western blot, neither crossing the use of specific (un)modified antigen/Ab. ELISA (inhibition assays) also confirmed this aspect.

As reported by Undas and colleagues [26], the *in vitro* modification of the proteins has the addition of Hcy-thiolactone at N-termini and ε-amines of lysines as an effect (referred as N-homocysteinylation). *In vivo*, proteins can undergo a transmethylation reaction, that is the



**Fig. 3.** Anti-native A1AT and anti-Hcy-A1AT Abs (anti-HATA) in patients and healthy subjects (HS). (A) Box-whisker plot of anti-native A1AT IgG in patients with (seropositive, SP-)RA, SN-RA, and from sex- and age-matched HS. Mean values of ODs and standard deviations (SDs) are indicated. The broken line represents the cut-off value (mean OD + 3 SDs for HS = 0.49). The occurrence of IgG Abs was significantly greater in RA patients with respect to HS ( $P < 0.0001$ ). (B) Box-whisker plot of anti-HATA IgG in patients with (SP-)RA, SN-RA, PsA, OA, and from sex- and age-matched HS. Mean values of ODs and SDs are indicated. The broken line represents the cut-off value (mean OD + 3 SDs for HS = 0.35). The occurrence of IgG Abs was significantly greater in patients with (SP-)RA, SN-RA, PsA and OA with respect to HS ( $P < 0.0001$  for all groups *versus* HS). Moreover, the occurrence in SN- and SP-RA (RA) patients groups was also significantly greater than in PsA and OA ( $P < 0.0001$ , respectively).



**Fig. 4.** Receiver operating characteristic (ROC) curves for anti-HATA. (A) SN-RA patients, and (B) whole RA patients' population *versus* HS. (C) SN-RA patients and (D) whole RA patients' population *versus* disease controls. The area under curve (AUC) from ROC analysis is 0.9204 (95% Confidence Interval, CI 0.8795–0.9613) for (A), 0.9517 (95% CI 0.9286–0.9748) for (B), 0.6539 (95% CI 0.5880–0.7199) for (C), 0.819 (95% CI 0.7754–0.8626) for (D).

conversion of methionine residues in homocysteine (t-homocysteinyl-lysine), but N-homocysteinyl-lysine is also possible. In this case, because of its similarity to the protein amino acid methionine, homocysteine is converted by methionyl-tRNA synthetase (MetRS) to homocysteinyl-adenylate. Homocysteine does not complete the protein biosynthetic pathway, but is edited by the conversion to homocysteine-thiolactone. Accumulation of homocysteine-thiolactone can be detrimental because of its ability to modify proteins. Indeed, human plasma levels of Hcy-proteins seem to be directly related to plasma t- and N-homocysteinyl-lysine levels, and autoAbs against N-ε-Hcy-Lys proteins are known to be present in human serum [26]. Beside the chronic inflammation as a causative factor, higher homocysteine levels in RA patients could be a consequence of drug assumption. This seems as a controversial aspect, since Rho and colleagues [27] found that homocysteine blood concentrations did not differ in patients receiving or not methotrexate, even if this might have been the result of concurrent folate administration, which is common practice in these patients. On the other hand, the study of Remuzgo-Martínez et al. [28] showed that patients with RA, in particular those with ischemic heart disease, displayed a decreased expression of the methylene tetrahydrofolate reductase gene, resulting in an elevated plasma level of homocysteine, which is considered as an independent risk factor for cardiovascular diseases. However, even though human blood proteins have been found to contain small amounts of homocysteine, this is a necessary but not a sufficient condition for developing an autoimmune response so, as well as for all autoimmune conditions, genetic predisposing factors are also needed.

In order to estimate the serum concentration of new autoAbs, we used the antigen directly isolated from SF by immunoprecipitation and the native protein *in vitro* modified by incubation with Hcy-thiolactone, to ensure that they were likewise both recognized from autoAbs in the sera, and the results obtained were virtually the same. Interestingly, we observed that a large proportion of SN-RA patients (75.7%), and also a very large number of SP-RA patients under testing (87.1%) displayed anti-HATA. Moreover, the percentage of SN-RA patients' sera with anti-HATA resulted even higher than those presenting serum anti-FCS CarP Abs. We found that a portion (54.5%) of SP-RA patients displayed Abs

against the native A1AT, which instead does not seem to be an antigenic target for SN-RA. We didn't find correlations between the presence of anti-HATA and disease activity (DAS28), neither with serological parameters (ESR, CRP). It is likely that a larger cohort of patients should be tested for a better characterization of these autoAbs. In a previous study, focused on the presence of Hcy-proteins as molecular targets [26], the existence of Abs anti-Hcy-hemoglobin and anti-Hcy-albumin in human serum was pointed out, suggesting Hcy-proteins as “neo-self” antigens. In this 16-year-old work, the authors also assessed that other novel self antigens identified in humans could have included proteins modified by oxidation or glycation, becoming this the groundwork for the study of the important role that PTMs would have played in inducing autoimmunity over the following years. Similarly to other diseases, PTMs may generate “new” epitopes that could mediate the ability of “modified self” to trigger autoimmunity. As a result, these altered pathways of autoantigen presentation may contribute to the perpetuation of chronic autoimmune diseases [29].

It is known that A1AT levels, associated with some A1AT genotypes, may be a contributory factor to RA development or tissue destruction [30]. Moreover, variation of the A1AT gene seems to be associated with increased ACPA production in RA, defining a distinct subset of patients with increased disease severity [31]. A1AT was already validated as one of the target proteins of the immune response in early-stage breast cancer [32]. Verheul and colleagues [33] found carbamylated A1AT being an antigenic target of anti-CarP antibodies in RA patients. In this study, the importance of A1AT in RA is well explained. A1AT is a protease inhibitor protein belonging to the serpin superfamily and protects tissues from the action of many enzymes produced by inflammatory cells, ranging between 1.5 and 3.5 g/liter in blood, concentration that can raise manifold upon acute inflammation. In its absence, neutrophil elastase is able to break down several molecules including elastin, an ubiquitous molecule present in connective tissue matrix, skin, ligaments, blood vessels, and lungs, in which contributes to the elasticity, resulting in respiratory complications, such as emphysema, or chronic obstructive pulmonary disease in adults and in cirrhosis. Beside these actions, A1AT might also exert other anti-inflammatory or tissue protecting effects, via dendritic cells or regulatory

T cells [30,34]. This aspect seems to be crucial for considering the role of A1AT in new treatment programs, which include most of the autoimmune diseases [35–37].

Preliminary data (not shown) revealed Hcy-A1AT expression at a cellular level, in lysates from both RA fibroblast-like synoviocytes and the immortalized human umbilical vein (endothelial) cell line EA.hy926. In view of the importance of the possible surface exposed epitopes of this antigen in autoimmunity (also in its post-translationally modified form), further studies are needed to confirm these data, especially for SN-RA patients, a disease subset in which is likely that pathogenic mechanisms underlying the disease may be different (and, so far, not well elucidated). So, in our opinion, protein expression at both synoviocyte and endothelium level might be a key factor, which could be involved in pathogenic mechanism of specific manifestations (articular and/or cardiovascular) of the disease.

In conclusion, our results obtained with both a proteomic and an immunological approach, prompt to identify Hcy-A1AT as a “new” antigenic target in RA. In this contest, we would also emphasize the importance of individuating a specific molecular target of autoAbs in RA. The presence of anti-HATA may be considered highly sensitive in these patients, and also quite specific, since they were detected only in 15% of patients with OA and in 25.2% of patients with PsA (in particular, this percentage is certainly lower, respect to those observed for RA patients, so that we can't firmly conclude that these autoAbs can play a role in PsA). Anyway, the individuation of these single-target autoAbs may represent a useful and specific tool for RA patients, and mainly in patients with clinical features suggestive of RA, in which the tests for detection of “classical” antibody specificities result continuously negative.

#### Author contributions

TC designed research study and conducted the experiments, collected, analyzed and interpreted data, wrote and revised the manuscript. DS, CM, AG and ADG performed the proteomic experiments and analyzed data. AP, FRS, LN, MS, CP, FC, AF, AIC and DC recruited patients and collected the clinical and demographic data. MV and CB helped in analyzing data. AA, MES and VB interpreted data and provided useful suggestions. FC and GV supervised the work, interpreted data and gave the final approval of the manuscript. CA designed the study, interpreted data and gave the final approval of the manuscript.

#### Declaration of competing interest

The Authors declare that they have no conflict of interest.

#### Acknowledgements

This research was supported by Progetto di Ateneo 2014 to Cristiano Alessandri, Sapienza University of Rome, Rome. Italy.

#### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jaut.2020.102470>.

#### Abbreviations

A1AT	Alpha 1 antitrypsin
Ab(s)	Antibody, antibodies
ACN	Acetonitrile
ACPA	Anti-citrullinated protein antibodies
ACR	American College of Rheumatology
Anti-FCS	CarP Abs Anti-fetal calf serum carbamylated proteins antibodies
AUC	Area under curve

AutoAb(s)	Autoantibody(ies)
CI	Confidence Interval
CRP	C reactive protein
DAS 28	Disease activity score 28
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-Linked ImmunoSorbent Assay
ESR	Erythrocyte sedimentation rate
HA	Human albumin
Hcy	Homocysteinylated
HLA	Human Leukocyte Antigen
HRP	Horseradish peroxidase
HS	Healthy subjects
HSE	Hyaluronidase
IAA	Iodoacetamide
IEF	Isoelectrofocusing
IgG	Immunoglobulin G
IP	Immunoprecipitated
IPG	Immobilized pH gradient
MALDI-TOF	Matrix Assisted Laser Desorption/Ionization-Time of Flight
MetRS	Methionyl-tRNA synthetase
MOWSE	MOlecular Weight SEarch
NanoLC	Nanoliquid chromatography
OA	Osteoarthritis
OD(s)	Optical density(ies)
PBS	Phosphate-buffered saline
PBS-T	PBS containing 0.05% Tween 20
PMF	Peptide mass fingerprint
PsA	Psoriatic arthritis
PTM(s)	Post-translational modification(s)
RA	Rheumatoid arthritis
RF	Rheumatoid factor
ROC	Receiver operating characteristic
SD(s)	Standard deviation(s)
SDS-PAGE	Sodium Dodecyl Sulphate-PolyAcrylamide Gel Electrophoresis
SF(s)	Synovial fluid(s)
SN-RA	Seronegative rheumatoid arthritis
SP-RA	Seropositive rheumatoid arthritis
TBS	Tris-buffered saline
TBS-T	TBS containing 0.1% Tween 20
2DE	Two (2) dimensional electrophoresis

#### References

- [1] D. Aletaha, T. Neogi, A.J. Silman, J. Funovits, D.T. Felson, C.O. Bingham III, N.S. Birnbaum, G.R. Burmester, V.P. Bykerk, M.D. Cohen, B. Combe, K.H. Costenbader, M. Dougados, P. Emery, G. Ferraccioli, J.M. Hazes, K. Hobbs, T.W. Huizinga, A. Kavanaugh, J. Kay, T.K. Kvien, T. Laing, P. Mease, H.A. Menard, L.W. Moreland, R.L. Naden, T. Pincus, J.S. Smolen, E. Stanislawski-Biernat, D. Symmons, P.P. Tak, K.S. Upchurch, J. Vencovsky, F. Wolfe, G. Hawker, Rheumatoid arthritis classification criteria: an American college of rheumatology/ European league against rheumatism collaborative initiative, *Arthritis Rheum.* 62 (2010) 2569e2581, <https://doi.org/10.1136/ard.2010.138461>.
- [2] C. Alessandri, F. Conti, P. Conigliaro, R. Mancini, L. Massaro, G. Valesini, Seronegative autoimmune diseases, *Ann. N. Y. Acad. Sci.* 1173 (2009) 52–59, <https://doi.org/10.1111/j.1749-6632.2009.04806.x>.
- [3] B. Nakken, G. Papp, V. Bosnes, M. Zeher, G. Nagy, P. Szodoray, Biomarkers for rheumatoid arthritis: from molecular processes to diagnostic applications-current concepts and future perspectives, *Immunol. Lett.* 189 (2017) 13–18, <https://doi.org/10.1016/j.imlet.2017.05.010>.
- [4] J. Shi, R. Knevel, P. Suwannaalai, M.P. van der Linden, G.M. Janssen, P.A. van Veelen, N.E. Levarht, A.H. van der Helm-van Mil, A. Cerami, T.W. Huizinga, R.E. Toes, L.A. Trouw, Autoantibodies recognizing carbamylated proteins are present in sera of patients with rheumatoid arthritis and predict joint damage, *Proc. Natl. Acad. Sci. U.S.A.* 108 (2011) 17372–17377, <https://doi.org/10.1073/pnas.1114465108>.
- [5] A. Pecani, C. Alessandri, F.R. Spinelli, R. Priori, V. Ricciari, M. Di Franco, F. Ceccarelli, T. Colasanti, M. Pendolino, R. Mancini, S. Truglia, C. Barbat, M. Vomero, D. Sabatinelli, F. Morello, G. Valesini, F. Conti, Prevalence, sensitivity



- and specificity of antibodies against carbamylated proteins in a monocentric cohort of patients with rheumatoid arthritis and other autoimmune rheumatic diseases, *Arthritis Res. Ther.* 18 (2016) 276, <https://doi.org/10.1186/s13075-016-1173-0>.
- [6] L.A. Trouw, T. Rispens, R.E.M. Toes, Beyond citrullination: other post-translational protein modifications in rheumatoid arthritis, *Nat. Rev. Rheumatol.* 13 (2017) 331–339, <https://doi.org/10.1038/nrrheum.2017.15>.
  - [7] M. Juarez, H. Bang, F. Hammar, U. Reimer, B. Dyke, I. Sahbudin, C.D. Buckley, B. Fisher, A. Filer, K. Raza, Identification of novel antiacetylated vimentin antibodies in patients with early inflammatory arthritis, *Ann. Rheum. Dis.* 75 (2016) 1099–1107, <https://doi.org/10.1136/annrheumdis-2014-206785>.
  - [8] A. Mastrangelo, T. Colasanti, C. Barbatì, A. Pecani, D. Sabatinelli, M. Pendolino, S. Truglia, L. Massaro, R. Mancini, F. Miranda, F.R. Spinelli, F. Conti, C. Alessandri, The role of post-translational protein modifications in rheumatological diseases: focus on rheumatoid arthritis, *J. Immunol. Res.* 2015 (2015) 712490, <https://doi.org/10.1155/2015/712490>.
  - [9] T. Colasanti, S. Fiorito, C. Alessandri, A. Serafino, F. Andreola, C. Barbatì, F. Morello, M. Alfè, G. Di Blasio, V. Gargiulo, M. Vomero, F. Conti, G. Valesini, Diesel exhaust particles induce autophagy and citrullination in Normal Human Bronchial Epithelial cells, *Cell Death Dis.* 9 (2018) 1073, <https://doi.org/10.1038/s41419-018-1111-y>.
  - [10] K.T. Tang, B.J. Tsuang, K.C. Ku, Y.H. Chen, C.H. Lin, D.Y. Chen, Relationship between exposure to air pollutants and development of systemic autoimmune rheumatic diseases: a nationwide population-based case-control study, *Ann. Rheum. Dis.* 78 (2019) 1288–1291, <https://doi.org/10.1136/annrheumdis-2019-215230>.
  - [11] R. Knevel, T.W.J. Huizinga, F. Kurreeman, Genomic influences on susceptibility and severity of rheumatoid arthritis, *Rheum. Dis. Clin. N. Am.* 43 (2017) 347–361, <https://doi.org/10.1016/j.rdc.2017.04.002>.
  - [12] K.D. Deane, M.K. Demoruelle, L.B. Kelmenson, K.A. Kuhn, J.M. Norris, V.M. Holers, Genetic and environmental risk factors for rheumatoid arthritis, *Best Pract. Res. Clin. Rheumatol.* 31 (2017) 3–18, <https://doi.org/10.1016/j.berh.2017.08.003>.
  - [13] L. Padyukov, M. Seielstad, R.T. Ong, B. Ding, J. Rönnelid, M. Seddighzadeh, L. Alfredsson, L. Klareskog, The Epidemiological Investigation of Rheumatoid Arthritis (EIRA) study group, A genome-wide association study suggests contrasting associations in ACPA-positive versus ACPA-negative rheumatoid arthritis, *Ann. Rheum. Dis.* 70 (2011) 259–265, <https://doi.org/10.1136/ard.2009.126821>.
  - [14] N.A. Doha, R.E. Toes, Rheumatoid arthritis: are ACPA-positive and ACPA-negative RA the same disease? *Nat. Rev. Rheumatol.* 7 (2011) 202–203 <https://www.nature.com/articles/nrrheum.2011.28>.
  - [15] F.R. Spinelli, A. Pecani, F. Ciciarello, T. Colasanti, M. Di Franco, F. Miranda, F. Conti, G. Valesini, C. Alessandri, Association between antibodies to carbamylated proteins and subclinical atherosclerosis in rheumatoid arthritis patients, *BMC Musculoskelet. Disord.* 18 (1) (2017) 214, <https://doi.org/10.1186/s12891-017-1563-8>.
  - [16] T. Bennike, U. Ayturk, C.M. Haslauer, J.W. Froehlich, B.L. Proffen, O. Barnaby, S. Birkelund, M.M. Murray, M.L. Warman, A. Stensballe, H. Steen, A normative study of the synovial fluid proteome from healthy porcine knee joints, *J. Proteome Res.* 13 (2014) 4377–4387, <https://doi.org/10.1021/pr500587x>.
  - [17] H. Liao, J. Wu, E. Kuhn, W. Chin, B. Chang, M.D. Jones, S. O'Neil, K.R. Clauser, J. Karl, F. Hasler, R. Roubenoff, W. Zolg, B.C. Guild, Use of mass spectrometry to identify protein biomarkers of disease severity in the synovial fluid and serum of patients with rheumatoid arthritis, *Arthritis Rheum.* 50 (2004) 3792–3803, <https://doi.org/10.1002/art.20720>.
  - [18] M.K. Kong, B.H. Min, P.C. Lee, Evaluation of a pretreatment method for two-dimensional gel electrophoresis of synovial fluid using cartilage oligomeric matrix protein as a marker, *J. Microbiol. Biotechnol.* 22 (2012) 654–658 <http://www.jmb.or.kr/journal/viewJournal.html?year=2012&vol=22&num=5&page=654>.
  - [19] M. Del Ben, D. Overi, L. Polimeni, G. Carpino, G. Labbadia, F. Baratta, D. Pastori, V. Noce, E. Gaudio, F. Angelico, C. Mancone, Overexpression of the vitronectin V10 subunit in patients with nonalcoholic steatohepatitis: implications for noninvasive diagnosis of NASH, *Int. J. Mol. Sci.* 19 (2018), <https://doi.org/10.3390/ijms19020603> pii E603.
  - [20] J. Perla, A. Undas, T. Twardowski, H. Jakubowski, Purification of antibodies against N-homocysteinylated proteins by affinity chromatography on N- $\omega$ -homocysteinyl-aminohexyl-agarose, *J. Chromatogr. B. Analyt. Technol. Biomed. Life. Sci.* 807 (2004) 257–261, <https://doi.org/10.1016/j.jchromb.2004.04.018>.
  - [21] J. Perla-Kaján, O. Stanger, M. Luczak, A. Ziółkowska, L.K. Malendowicz, T. Twardowski, S. Lhotak, R.C. Austin, H. Jakubowski, Immunohistochemical detection of N-homocysteinylated proteins in humans and mice, *Biomed. Pharmacother.* 62 (2008) 473–479, <https://doi.org/10.1016/j.biopha.2008.04.001>.
  - [22] H. Jakubowski, Protein homocysteinylolation: possible mechanism underlying pathological consequences of elevated homocysteine levels, *Faseb. J.* 13 (1999) 2277–2283, <https://doi.org/10.1096/fasebj.13.15.2277>.
  - [23] R. Glowacki, H. Jakubowski, Cross-talk between Cys34 and lysine residues in human serum albumin revealed by N-homocysteinylolation, *J. Biol. Chem.* 279 (2004) 10864–10871 <http://www.jbc.org/content/279/12/10864>.
  - [24] T. Colasanti, M. Vomero, C. Alessandri, C. Barbatì, A. Maselli, C. Camperio, F. Conti, A. Tinari, C. Carlo-Stella, L. Tuosto, D. Benincasa, G. Valesini, W. Malorni, M. Pierdominici, E. Ortona, Role of alpha-synuclein in autophagy modulation of primary human T lymphocytes, *Cell Death Dis.* 5 (2014) e1265, <https://doi.org/10.1038/cddis.2014.211>.
  - [25] T. Colasanti, C. Alessandri, A. Capozzi, M. Sorice, F. Delunardo, A. Longo, M. Pierdominici, F. Conti, S. Truglia, A. Siracusano, G. Valesini, E. Ortona, P. Margutti, Autoantibodies specific to a peptide of  $\beta$ 2-glycoprotein I cross-react with TLR4, inducing a proinflammatory phenotype in endothelial cells and monocytes, *Blood* 120 (2012) 3360–3370, <https://doi.org/10.1182/blood-2011-09-378851>.
  - [26] A. Undas, J. Perla, M. Lacinski, W. Trzeciak, R. Kaźmierski, H. Jakubowski, Autoantibodies against N-homocysteinylated proteins in humans: implications for atherosclerosis, *Stroke* 35 (2004) 1299–1304, <https://doi.org/10.1161/01.STR.0000128412.59768.6e>.
  - [27] Y.H. Rho, A. Oeser, C.P. Chung, G.L. Milne, C.M. Stein, Drugs used in the treatment of rheumatoid arthritis: relationship between current use and cardiovascular risk factors, *ADI* 2 (2009) 34–40, <https://doi.org/10.1111/j.1753-5174.2009.00019.x>.
  - [28] S. Remuzgo-Martínez, F. Genre, R. López-Mejías, B. Ubilla, V. Mijares, T. Pina, A. Corrales, R. Blanco, J. Martín, J. Llorca, M.A. González-Gay, Decreased expression of methylene tetrahydrofolate reductase (MTHFR) gene in patients with rheumatoid arthritis, *Clin. Exp. Rheumatol.* 34 (2016) 106–110 <https://europepmc.org/article/med/26843177>.
  - [29] H.A. Doyle, M.L. Yang, M.T. Raycroft, R.J. Gee, M.J. Mamula, Autoantigens: novel forms and presentation to the immune system, *Autoimmunity* 47 (2014) 220–233, <https://doi.org/10.3109/08916934.2013.850495>.
  - [30] D.W. Cox, O. Huber, Rheumatoid arthritis and alpha-1-antitrypsin, *Lancet* 1 (1976) 1216–1217, [https://doi.org/10.1016/S0140-6736\(76\)92163-2](https://doi.org/10.1016/S0140-6736(76)92163-2).
  - [31] C. McCarthy, C. Orr, L.T. Fee, T.P. Carroll, D.M. Dunlea, D.J.L. Hunt, E. Dunne, P. O'Connell, G. McCarthy, D. Kenny, U. Fearon, D.J. Veale, E.P. Reeves, N.G. McElvaney, Brief Report: genetic variation of the  $\alpha$ 1-antitrypsin gene is associated with increased autoantibody production in rheumatoid arthritis, *Arthritis Rheum.* 69 (2017) 1576–1579, <https://doi.org/10.1002/art.40127>.
  - [32] E. López-Árias, A. Aguilar-Lemarroy, L. Felipe Jave-Suárez, G. Morgan-Villela, I. Mariscal-Ramírez, M. Martínez-Velázquez, A.H. Alvarez, A. Gutiérrez-Ortega, R. Hernández-Gutiérrez, Alpha 1-antitrypsin: a novel tumor-associated antigen identified in patients with early-stage breast cancer, *Electrophoresis* 33 (2012) 2130–2137, <https://doi.org/10.1002/elps.201100491>.
  - [33] M.K. Verheul, A. Yee, A. Seaman, G.M. Janssen, P.A. van Veelen, J.W. Drijfhout, R.E.M. Toes, M. Mahler, L.A. Trouw, Identification of carbamylated alpha 1 antitrypsin (A1AT) as an antigenic target of anti-CarP antibodies in patients with rheumatoid arthritis, *J. Autoimmun.* 80 (2017) 77–84, <https://doi.org/10.1016/j.jaut.2017.02.008>.
  - [34] M. Gatto, L. Iaccarino, A. Ghirardello, N. Bassi, P. Pontisso, L. Punzi, Y. Shoenfeld, A. Doria, Serpins, immunity and autoimmunity: old molecules, new functions, *Clin. Rev. Allergy Immunol.* 45 (2013) 267–280, <https://doi.org/10.1007/s12016-013-8353-3>.
  - [35] D.A. Bergin, K. Hurley, N.G. McElvaney, E.P. Reeves, Alpha-1 antitrypsin: a potent anti-inflammatory and potential novel therapeutic agent, *Arch. Immunol. Ther. Exp.* 60 (2012) 81–97, <https://doi.org/10.1007/s00005-012-0162-5>.
  - [36] E. Ortona, A. Maselli, F. Delunardo, T. Colasanti, A. Giovannetti, M. Pierdominici, Relationship between redox status and cell fate in immunity and autoimmunity, *Antioxidants Redox Signal.* 21 (2014) 103–122 <https://www.liebertpub.com/doi/10.1089/ars.2013.5752>.
  - [37] D. Crossley, R. Stockley, E. Sapey, Alpha-1 antitrypsin deficiency and accelerated aging: a new model for an old disease? *Drugs Aging* 36 (2019) 823–840 <https://link.springer.com/article/10.1007%2Fs40266-019-00684-7>.