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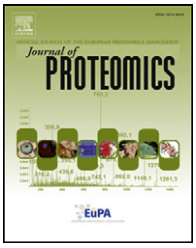

Discovery of serum proteomic biomarkers for prediction of response to infliximab (a monoclonal anti-TNF antibody) treatment in rheumatoid arthritis: An exploratory analysis

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ABSTRACT

Biologics such as TNF antagonists are a new class of drugs that have greatly improved Rheumatoid Arthritis (RA) treatment. However, for unknown reasons, individual patients with RA respond to one of these drugs but not to others even those targeting the same molecule. Methods to predict response are sorely needed because these drugs are currently selected by trial and error, what is very inefficient and prejudicial for the patient and the healthcare system. Here, we have explored the discovery of protein biomarkers in serum from patients treated with infliximab, one of the major anti-TNF drugs. The study was based in a quantitative proteomics approach using 8-plex iTRAQ labeling. It combined depletion of the most abundant serum proteins, two-dimensional LC fractionation, protein identification and relative quantification with a hybrid Orbitrap mass spectrometer. This approach allowed the identification of 315 proteins of which 237 were confidently quantified with two or more peptides. The detection range covered up to 6 orders of magnitude including multiple proteins at the ng/mL level. A new set of putative biomarkers was identified comprising 14 proteins significantly more abundant in the non-responder patients. The differential proteins were enriched in apolipoproteins, components of the complement system and acute phase reactants. These results show the feasibility of this approach and provide a set of candidates for validation as biomarkers for the classification of RA patients before the beginning of treatment, so that anticipated non-responders could be treated with an alternative drug.

1. Introduction

Rheumatoid arthritis (RA) is a chronic systemic autoimmune disease of complex etiology comprising genetic and environmental factors that is characterized by inflammation in multiple joints [1]. Left without treatment, it progresses to disability, deformities due to bone erosion and life shortening. RA prevalence is about 1% of the world population. Classical

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treatments are still commonly used, but they are not sufficiently effective for many patients. In the last decade, new drugs became available in the group of biologics (monoclonal antibodies, soluble receptors or other complex molecules targeting specific players in the disease process). The first that were available for RA treatment were the tumor necrosis factor (TNF) antagonists. Among them, infliximab, a chimeric antibody comprising a human IgG1 constant fraction and a murine variable region targeting membrane and soluble TNF [2], has become one of the biologics most commonly used in RA.

Biologics have greatly improved RA treatment but none of them is effective in all patients. For unknown reasons, about a third of the patients in whom one of these drugs is assayed fail to show significant improvement. These patients can respond to an alternative biologic targeting the same or a different molecule [3]. Currently, clinical or laboratory methods for the prediction of patients response are not available. Therefore, the only approach to select biologics for a particular RA patient is by trial and error. This approach is associated with notable inefficiency and prejudices because responsiveness can only be assessed after three to six months of treatment. During this time, patients suffer uncontrolled disease with the potential of irreversible damage, and the healthcare system expends large amounts in ineffective drugs. Thus, it is necessary to find biomarkers that make possible the identification of non-responder patients in advance, to treat them with an alternative drug from the beginning. Many studies have already tried to identify this type of biomarkers in the genetics, functional genomics, proteomics, autoantibody and clinical fields, but no reproducible and informative findings have yet been reported [4].

Very few proteomic studies have attempted to identify biomarkers for prediction of response to biologics in RA. A couple of studies analyzed selected cytokines or cytokines plus RA autoantibodies showing that some of them were associated with clinical response to the TNF antagonist etanercept [5,6]. By contrast, not a single cytokine was associated with response to a different biologic, rituximab, in a similar study [7]. The unique previous agnostic proteomic study was done by Trocmé et al. [8]. These authors used SELDI-TOF technology to identify plasma biomarkers for prediction of response to infliximab. Six potential biomarkers were detected, although only two proteins were identified. None of the previous studies have been independently replicated. This paucity of proteomic research on biomarkers for prediction of response contrasts with the multiple studies searching RA diagnostic and prognostic biomarkers [9–20], and with several proteomic studies monitoring the changes that take place after the administration of different drugs [7,16,21,22].

Isobaric Tags for Relative and Absolute Quantitation (iTRAQ) is a quantitative proteomic approach ideally suited for biomarker discovery. It provides quantification, identification and multiplexing in a single assay. However, it has been scarcely used to study human serum and plasma. This is unfortunate because serum and plasma are informative for many diseases, especially for systemic diseases like RA, and easily available. The latter is very important when validating the potential biomarkers and also for their future widespread use. For example, patients with RA are not routinely subjected to synovial tissue biopsies and it will pose significant difficulties to implement them for drug selection. Unfortunately, discovery of biomarkers among the serum or plasma proteins is limited by their great complexity and wide dynamic range. Protein concentrations extend for more than 11 orders of magnitude with the top 10 most abundant plasma proteins accounting for ~90% of the total proteins [23]. Disease biomarkers are usually present at low concentrations (~ ng/mL) [23], being masked by higher abundance proteins in 2-DE and being blurred in MS due to competitive ionization and signal suppression. Therefore, the quantitative and qualitative analysis of low abundance proteins is challenging. To overcome these problems, there is a need for (i) prefractionation methods to specifically remove the high abundance proteins; (ii) good separation techniques to further decrease protein complexity; and (iii) MS equipments with high sequencing speed and sensitivity. In this study, we have explored the performance of an approach including these characteristics. It was applied to the discovery of biomarkers for prediction of response in serum of patients with RA that had been prospectively evaluated during treatment with infliximab. The proteomics approach comprised an immunodepletion prefractionation step, a thorough 2-D LC fractionation and quantification of the differentially isotopic labeled peptides by MS/MS. Results were satisfactory because this approach allowed the identification of a large number of proteins, covering a wide dynamic range and including many proteins in the ng/mL level, and identifying 14 putative biomarkers for prediction of response to infliximab that are consistent with our knowledge of the disease.

2. Materials and methods

2.1. *Ethics approval*

The project was approved by the Ethics Committee for Clinical Research of Galicia and carried out according to the Helsinki Declaration Principles. All participating subjects gave their written informed consent.

2.2. *Sample collection*

Patients with RA according to the American College of Rheumatology (ACR) classification criteria [24] from a single center (Gregorio Marañón Hospital, Madrid, Spain) were enrolled in the study. All were naive for any biologics before the start of the enrollment period. Blood was collected into 8 ml Vacuette Z Serum Sep Clot Activator tubes (Greiner Bio-One GmbH, Frickenhausen, Germany) before starting infliximab administration, left to clot at room temperature for 2 h and then centrifuged at 3000*g* for 10 min. The collected serum was aliquoted and stored at −80 °C. Infliximab (Remicade; Centocor Inc., Malvern, PA) was given following the standard dose and administration schedule. Clinical response was determined 6 months after infliximab initiation according with the European League Against Rheumatism (EULAR) criteria based in the Disease Activity Score 28 (DAS28) [25]. Only patients classified