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ORIGINAL ARTICLE

The Bioefficacy Protocol: biomarkers identification of TNF inhibitors efficacy in axial spondyloarthritis patients using transcriptome and proteome analysis

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ABSTRACT

Background: Axial Spondyloarthritis (axSpA) is a chronic inflammatory rheumatic condition affecting the axial skeleton, leading to pain, stiffness, and fatigue. While biologic therapies have improved clinical management, many patients experience partial or no responses, resulting in delays in disease control. Additionally, the risk of adverse events and increased costs remains a concern.

Objectives: Our primary objectives are: 1. to identify reliable markers for treatment response to Tumor Necrosis Factor alpha inhibitors (TNFi), in particular Adalimumab, enabling the identification of individuals most likely to benefit; 2. to analyze the impact of TNFi on gene and protein expression.

Methods: A multicenter, prospective 14-week study will be conducted with 36 participants aged 18-75 years, meeting the ASAS criteria for axSpA. Patient enrollment will follow the National Guidelines for the use of TNFi in axSpA treatment, with all included patients using TNFi (Adalimumab) as a first-line option.

Epidemiological and clinical data will be collected, along with peripheral blood samples, for integrated transcriptome, using RNA Seq (whole genome sequencing) and proteome analysis at various time points (baseline, 3-5 days, weeks 2 and 14), corresponding to the initial administration of TNFi. Patients will be classified as responders and non-responders, primarily based on ASAS20 criteria and secondarily based on ASDAS-C Reactive Protein (CRP), at week 14.

Discussion: This project's innovative approach lies in identifying potential biomarkers for TNFi (Adalimumab) response at baseline, paving the way for advancements in precision medicine in this field. Additionally, it seeks to establish evidence of the therapy's impact on gene and protein expression, offering deeper insights into the pathophysiological mechanisms underlying the therapeutic response.

Keywords: Spondyloarthritis; Adalimumab; Therapeutic Response Biomarkers; Transcriptome; Proteome.

KEY MESSAGES

- Identification of potential biomarkers TNFi response at baseline, in axSpA patients.
- Establishment of evidence for the therapeutic impact on gene and protein expression, in axSpA patients.
- Deeper understanding of the physiopathologic mechanisms associated with the therapeutic response, in axSpA patients.

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INTRODUCTION

Radiographic axial spondyloarthritis (r-axSpA), formerly known as Ankylosing Spondylitis (AS), is characterized by its association with *HLA-B27* and a predisposition to affect the axial skeleton, particularly the spine and pelvis. This condition is driven by inflammation at the entheses and manifests predominantly as inflammatory back pain. The hallmark of r-axSpA is the formation of new bone, including syndesmophytes and ankylosis[1]. Spondyloarthritis, as a whole, represents some of the most forms of inflammatory arthritis, with a reported prevalence of up to 1.6%, exceeding the 0.6% prevalence of rheumatoid arthritis[2].

axSpA typically begins at a relatively early age, often affecting young individuals around 30 years old, and leads to decline in physical function and quality of life. Work disability is prevalent among these patients, which has a significant impact in productivity and social costs.

axSpA is associated with a 50% increase in mortality compared to age- and gender- matched controls[2].

The progressive ankylosis of affected joints is currently irreversible, making early diagnosis and treatment essential for achieving the best prognosis. Numerous studies have indicated an average delay of at least 8 years between the onset of symptoms and a definitive diagnosis, which in turn delays the initiation of effective therapy. This delay is critical to consider when evaluating the potential for clinical harm[3,4,5].

The introduction of biologic therapies, such as TNFi, has revolutionized clinical practice, offering significant advantages in both management and prognosis. Although many axSpA patients respond well to these therapies, partial responses remain relatively common[6]. Identifying reliable predictors of treatment response would provide substantial clinical benefits by enabling more precise targeting of these therapies to those most likely to benefit. However, it is crucial to validate the discriminative capacity of these biomarkers in routine clinical practice.

The Ankylosing Spondylitis Disease Activity Score (ASDAS) has become a valuable tool for assessing disease activity. However, despite its utility in clinical practice, it lacks predictive capability for treatment response[2]. Current response predictors, such as younger age, *HLA-B27* positivity, elevated acute-phase reactants (CRP), and significant spinal inflammation on MRI, are associated with better outcomes[7]. Conversely, factors like older age, structural damage, and impaired function may predict suboptimal or non-response[8,9]. Although these markers hold statistical significance, their clinical discriminatory power remains limited. This underscores the urgent need for novel and more precise biomarkers.

In recent years, advancements in genotyping and study design have revolutionized the field of diseases with genetic predisposition, including axSpA, where several genes, beyond HLA-B27, have been implicated. These discoveries provide valuable insights into the pathogenesis of r-axSpA[10]. Moreover, studies have shown that TNFi treatment induces significant changes in gene expression and in proteomic profiles, highlighting the potential of systematic gene expression and proteome analysis to uncover new pathogenic pathways relevant to axSpA inflammation[11,12] and, potentially, therapeutic response. Integrating transcriptomic and proteomic profiling enhances the robustness of emerging data, reinforcing the value of these techniques in exploring disease mechanisms and therapeutic responses.

STUDY AIMS

Primary objective

The primary objective of The Bioefficacy study is to identify blood serum biomarkers for that can predict the response to TNFi (Adalimumab) therapy in axSpA patients (r-axSpA and non-radiographic axSpA (nr-axSpA). This will be accomplished by discovering new candidate genes and proteins with differential expression between responders and non-responders at various time points, using ribonucleic acid (RNA) sequencing (RNA Seq) and liquid chromatography with tandem mass spectrometry (LC-MS/MS methodologies)[13].

Secondary objectives

1. To assess changes in gene and proteins expression over a 14-weeks period of TNFi (Adalimumab) treatment.
2. To compare biomarkers identification based on different treatments outcomes (ASAS20 *vs* ASDAS CRP response- clinically important improvement, Δ>1,1).
3. To evaluate the impact on quality of life using the Ankylosing Spondylitis Quality of Life Questionnaire (ASQOL) and Short-Form Health Survey (SF-36) questionnaires throughout the 14-week TNFi therapy regimen[14].

METHODS

The Bioefficacy study - Biomarkers Identification of TNFi Efficacy in Ankylosing Spondylitis Patients Using Transcriptome Analysis and Mass Spectrometry (clinical trials.gov identifier NCT02492217) has been approved by the respective hospital boards and the national authorities, including the National Ethics Committee for Clinical Research (CEIC) and INFARMED (the competent authority regulating medicinal products). The study will be conducted in accordance with Good Clinical Practices (GCP)[15] guidelines, the Declaration of Helsinki[16], and all applicable legal regulations, ensuring participant safety and data protection mechanisms.

Written informed consent will be obtained from all participants prior to inclusion. This is a multicenter, prospective, observational, 14-week study involving adult axSpA patients meeting the ASAS criteria[17].

Patient population

To have 80% power to detect a 0.5SD difference response to TNFi between groups at p=0.05 (paired t-test), we estimated that we would need samples from 18 responders and 18 non-responders. Usually only 60% of patients after starting TNFi reach ASAS20[13] which means that we would need to include a larger number of patients to establish the subgroups for analysis. Thus, we will include the number of patients necessary

to ensure 18 non-responders, after which we will close the recruitment period. All clinical evaluations will be performed by previously trained rheumatologists.

Inclusion criteria

- axSpA according to ASAS criteria;
- Patient enrolment followed National Guidelines for TNFi use for the treatment of axSpA[14];
- Adults between 18 to 75 years;
- Ability to provide informed consent;
- Corticosteroid (equivalent to ≤ 10 mg prednisone) and/or NSAID therapy allowed considering a stable dose during 4 weeks before the study initiation;
- Adequate contraception in men (barrier) and women (barrier or hormonal) of childbearing age potential (patients and their partners);
- Adequate renal and hepatic function (values until 2 times above the normal level were allowed).

Exclusion criteria

- Previous treatment with bDMARDs;
- Intraarticular (axial or peripheral) joints or extra-articular injections within 28 days before screening;
- Current pregnancy or breastfeeding;
- History of rheumatic disorder other than axSpA;
- Any uncontrolled medical condition (e.g., diabetes mellitus, unstable ischemic heart disease, others…);
- History or signs of demyelinating disease;
- Active or latent tuberculosis or histoplasmosis;
- Malignancy (except for completely treated squamous or basal cell carcinoma);
- Positive serology for hepatitis B, hepatitis C, or human immunodeficiency virus;
- Any persistent or severe infection within 30 days before screening;
- Ankylosis of the total spine (syndesmophytes presence at all levels from D12 to S1 in X- ray lumbar spine lateral view).

Eligible patients will be recruited at the Portuguese Institute of Rheumatology, Lisboa; Spondyloarthritis Clinic of Centro Hospitalar Lisboa Ocidental, Hospital de Egas Moniz, Lisboa; Rheumatology Department, Centro Hospitalar e Universitário de São João, Porto; Rheumatology Department, Hospital Vila Nova de Gaia/Espinho, Vila nova de Gaia; Rheumatology Department, Unidade Local de Saúde do Alto Minho, Ponte de Lima; Rheumatology Department, Centro Hospitalar e Universitário de Coimbra; Rheumatology Department, Centro Hospitalar Médio Tejo, Torres Novas.

Patients, above 18 and below 75 years old, with clinical criteria for TNFi therapy according to Portuguese Society of Rheumatology guidelines will be treated with adalimumab 40 mg/0.8 mL subcutaneously, every other week, for 14 weeks.

CLINICAL AND EPIDEMIOLOGICAL CHARACTERIZATION AND COLLECTION OF BIOLOGICAL SAMPLES

All participants will be characterized through a web based regulated questionnaire, the Rheumatic Diseases Portuguese Register (Reuma.Pt)[18] developed by the Portuguese Society of Rheumatology. Clinical evaluations and peripheral blood collections will be performed at baseline (D0), and after 3-5 days (D3), 2 weeks (W2) and 14 (W14) weeks, corresponding to the initial administration of TNFi, from all participants.

Data collection allows the patients characterization in terms of: (1) Clinical data (D0) - demographic data, age at symptom onset, disease duration, systemic associated symptoms, disease patterns and previous therapeutic use; (2) Disease activity (D0, D3, W2, W14) - assessed by Bath Ankylosing Spondylitis Disease Activity Index (BASDAI), ASAS20 and ASDAS ESR, ASDAS CRP; (3) Functional Status (D0, D3, W2, W14) - assessed by Bath Ankylosing Spondylitis Functional Index (BASFI) and by the Bath Ankylosing Spondylitis Metrology Index (BASMI); (4) Quality of Life (QoL) data (D0, W14) - assessed by SF-36, European Quality of Life Questionnaire (EuroQol) and Health Assessment Questionnaire for AS (HAQ-AS); (5) Imaging data – The X-rays assessed by the modified Stoke Ankylosing Spondylitis Spine Score (mSASSS) (D0) and the MRI by the Berlin method (19)(D0, W14); (6) Blood samples will be collected at baseline (D0) to test for HLA-B27 status and at each timepoint (D0, D3, W2, W14) to assess inflammatory parameters (erythrocyte sedimentation rate (ESR) and CRP), for proteomic analysis and RNA-Seq (PAXgenes Blood RNA Sysem® (PreAnalytiX, QIAGEN) tubes, handling and storage according to the manufacturer's recommendations. Patients will be classified as responders and non-responders, primary according to ASAS20(20)(21) and secondary according to ASDAS CRP, at week 14.

Transcriptome and proteome data collection

We will screen peripheral blood samples to compare gene/protein signatures. Such data will aim to discover new candidate genes and proteins that are differentially abundant in responders' vs non-responders´ patients to TNFi therapy. In addition, these results will inform about the biological pathways associated with the TNFi response.

Global transcriptome analysis by RNA sequencing

Total RNA will be extracted from whole blood samples according to the standard PAXgene protocol (Qiagen,

2008)[22]. The quantity of RNA will be measured using a NanoDrop 2000/2000c Spectrophotometer according to the manufacturer's procedure (Thermo-Scientific, 2000); RNAs with a 260:280 ratio of ≥1.5 will be sequenced as below. The quality and quantity of the libraries will be assessed by the Fragment Analyzer with the method of DNF-474-22 - HS NGS Fragment 1-6000bp (Agilent). Sequencing library preparation will be performed using Illumina TruSeq stranded mRNA library preparation kits, with 100ng of total RNA as input. Libraries will be sequenced on an Illumina NextSeq500 sequencer (average of 39 million reads per sample, 75 base-pair paired-end). Sample correspondence between timepoints will be confirmed using SmaSH[23]. Raw sequencing reads will be aligned to gencode (v32) transcripts using kallisto (version 0.46.1)[24]. The edge R package will be used to filter low-expressed genes with the filterByExpr function and to normalize raw counts with the trimmed mean of M-values (TMM) normalization approach[25].

Proteome analysis by mass spectrometry (LC-MS/MS)

The proteomic experimental workflow will comprise the immunoaffinity depletion of the 6-interfering high-abundant proteins (albumin, IgG, IgA, haptoglobin, α-1-antitrypsin and transferrin) from human plasma using the Multiple Affinity Removal Spin Cartridge Human 6 Kit (Agilent Technologies®). This step will be followed by the tryptic digestion of the proteins extracted from the depleted serum, HPLC separation of the tryptic peptides, MS/MS-based identification, and MS quantification of the detected proteins. The LC-MS/MS analysis will be performed with a Q-Exactive™ HF high resolution tandem mass spectrometer (ThermoFisher Scientific™) incorporating an ultra-high-field Orbitrap analyser as previously described[26]. A bioinformatic analysis will be performed where all peptide matches with a peptide score below a query identity threshold *p*-value of 0.05 will be filtered and assigned to proteins. A protein identification will be deemed as valid when at least two different non-ambiguous peptides (i.e. not present in another polypeptide from the database) are detected in the whole dataset. For each validated protein, the number of MS/MS spectra for all detected non-ambiguous peptides or 'Spectral Count'[27] will be used as a proxy of their abundances[28].

Data Analysis

Descriptive statistics will be used to summarize baseline characteristics for responders and non-responders. Two sample Wilcoxon tests (continuous variables) and chi-square tests of association (categorical variables) will be used to compare characteristics between responders and non-responders at different timepoints, in particular between baseline and week 14.

Differential gene and protein abundance analysis will use the limma R package[29] to apply a voom transformation for variance stabilization on normalized expression values, and to obtain differentially expressed genes through an empirical bayes approach, followed by multiple test correction with the Benjamini-Hochberg method[30]. Genes and proteins will be considered differentially abundant if the adjusted *p*-value of the test is less than 0.05. The evaluation performed at D3 will permit to detect how early anti TNFi therapy may induce changes in gene and protein abundance.

Logistic regression models, and plotting will be performed using the R software. Sparse partial least squares discriminant analysis (sPLS-DA)[31] will be performed using the mixOmics R package[32]. Random forest models will be obtained using the randomForest R package.

DISCUSSION

The advent of TNFi has significantly improved the clinical management and prognosis of axSpA, particularly in cases where conventional therapies have yielded suboptimal results[33]. Despite the proven effectiveness of TNFi in approximately 60% of axSpA patients[34] a substantial proportion still do not respond adequately. This situation results in a considerable number of patients undergoing ineffective therapy, with potential adverse events and significant societal costs. Given the uncertainty surrounding treatment outcomes, this study aims to identify early blood serum molecular biomarkers capable of distinguishing between responders and non-responders among axSpA patients, as evaluated by ASAS20, after a 14-week treatment period. We propose a comprehensive, multidisciplinary, and cutting-edge study that facilitates an in-depth exploration of blood serum biomarkers through transcriptome and proteome approaches, all conducted on the same group of participants.

While numerous studies have investigated the overall impact of TNFi treatment on axSpA, allowing the identification of genes linked to immunity and inflammation[35,11,36] none have specifically delved into the molecular alterations associated with the response/non-response to TNFi treatment. The studies realized with this objective focused on specific markers of inflammation using a limited set of patients. Notably, no study has consistently evaluated large-scale transcriptome and/or proteome data to uncover early predictors of TNFi response in axSpA.

In this light, our work may represent a key contribution to the discovery of new biomarkers to assess the

potential response to TNFi (adalimumab) before therapy is initiated and additionally to explain the molecular basis of TNFi response and eventually mechanistic information on disease progression.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This study (registered in clinical trials.gov with the identifier NCT02492217) was approved by the national competent authorities: National Ethics Committee for Clinical Research (CEIC) and INFARMED (competent authority to regulate medicinal products). The study will be conducted according to Good Clinical Practices (GCP), Declaration of Helsinki, and legal regulation applicable, ensuring participants' safety and mechanisms for data protection. Written informed consent will be obtained from all participants before study inclusion. None of the patient identifiers will be known to anyone outside of the research group. There was no active involvement of patients or the public as co-producers of research in this project. However, all participants will be invited to participate in a meeting where the main results will be presented and discussed.

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