DISCOVERY AND CLINICAL USEFULNESS OF BLOOD BIOMARKERS IN STROKE-ASSOCIATED PNEUMONIA

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1. Abstract

Stroke-associated pneumonia (SAP) represents one of the most frequent and severe post-stroke complications, leading to high rates of mortality and disability, as well as high costs and hospital stays for those patients who develop it.

Nowadays, no preventive therapies have been demonstrated to reduce the risk of SAP in stroke patients. Moreover, SAP diagnosis is not easy for clinicians. Even at the research level, diagnostic criteria for SAP are not uniform among studies. Our hypothesis was that a more accurate selection of patients at the highest risk for this complication would identify a population that might benefit in the future from specific therapeutic measures. With this background, the main objective of this project was to identify, by different approaches, blood biomarkers able to predict the development of this complication. In addition, the secondary objective of this project was to improve SAP diagnosis by validating the modified CDC diagnostic criteria, proposed by a group of experts in 2015, and assessing the role of high-resolution chest CT for the diagnostic confirmation of PAS.

To fulfill these objectives, we carried out a prospective, multicenter study in 8 stroke units from Spain, which included a total of 340 stroke patients within the first 24 hours after stroke onset. We collected two blood samples in those patients; the first sample within the first 24 hours, and a second sample 24 hours later. From these blood samples, we obtained serum, plasma and RNA for the study of blood biomarkers. In addition, in a subgroup of 120 patients, we isolated three leukocyte subpopulations (CD3+, CD14+, CD16+), from which we obtained RNA and proteins for discovery studies. All patients underwent a daily clinical follow-up for seven days after hospital admission, to determine the appearance of pneumonia. Later, three months after stoke, functional status was also assessed. In addition, in a subgroup of patients with severe strokes, a high-resolution chest CT was performed between the 5th and 7th days after the stroke. The study enrolment was conditioned by the impact of the COVID19 pandemic, which led to interrupt recruitment for three months, and later to delayed inclusion rate.

Biomarkers were determined from different points of view. First, in a cohort of 28 patients, of whom 14 developed SAP, known biomarkers previously described by the

group were assessed, as well as others described in the literature. The best discriminatory biomarkers were later measured in the 340 patients included in the entire cohort. In addition, proteomics and transcriptomics experiments in the three isolated subpopulations were performed in a subgroup of 12 patients (6 with SAP, 6 without SAP), from which the top candidates were selected to be evaluated in a larger cohort of 22 patients, either by real-time PCR (RT-PCR, for RNA) and western-blot (WB, for proteins), in the same subpopulations. The best candidates from these experiments were validated at the protein level in peripheral blood samples in a cohort of 28 patients (at both time-points) using ELISA.

2. Results

The four objectives included in the initial project are here summarized in two.

OBJECTIVE 1. Biomarkers discovery and validation

By proteomic and transcriptomic techniques in the different leukocyte subpopulations, lymphocytes (CD3+), monocytes (CD14+) and neutrophils (CD16+), we identified that neutrophils were the subpopulation expressing more changes between patients developing SAP or not. Moreover, bioinformatic analysis of these results showed that the most impaired biological processes related to SAP occurrence were neutrophil degranulation and granulocyte activation. From this experiment, a total of three candidate proteins from the proteomics experiment, and 12 candidate genes from the transcriptomics experiment, were selected for further validation in leukocyte subpopulations from new samples, by using RT-PCR and WB. Unfortunately, we were not able to identify the three candidates coming from the neutrophil transcriptomics experiment by RT-PCR. Of the remaining candidates, YKL-40 and PGPRS (neutrophil proteomics), S100A8 and S100A12 (lymphocyte transcriptomics), adrenomedullin (monocyte transcriptomics) and GADD45A (whole blood transcriptomics) showed results in the same line as in the discovery experiment. In the final validation, by ELISA in peripheral blood samples, YKL-40 and S100A12 showed significant differences between patients with and without SAP.

A panel of 9 candidate biomarkers, from the literature and from previous results of the group, were explored in 38 patients at two time points. From this experiment, three candidates (CRP, MR-proADM and SAA) were selected and evaluated in the entire

patient cohort (n=340). All three biomarkers showed higher levels in patients with SAP at both time points. The three-biomarker panel showed a good predictive value for SAP, especially at the second time point. In fact, the panel showed a sensitivity of 83% and a specificity of 60% within the first 24 hours (time 1), and a sensitivity of 91% and a specificity of 76% at 24-48 hours (time 2), respectively. The inclusion of this panel in logistic regression models, together with clinical variables, resulted in an increased predictive ability, increasing areas under the ROC curves from 0.77 to 0.86 (time 1), and from 0.86 to 0.92 (time 2).

OBJECTIVE 2. Improving SAP clinical diagnosis

In this objective, we evaluated the usefulness of the modified CDC criteria for SAP diagnosis, as well as the adherence to these criteria in current clinical practice, in terms of SAP diagnosis and treatment initiation. Of the 340 included patients, 72 (21.8%) developed an infection, of which 38 (11.5%) were diagnosed as pneumonia by clinicians. Of these cases, 24 (7.3%) met the modified CDC criteria for SAP. Antibiotics were used in 92% of the patients who met the modified CDC criteria and in 50% of those who did not. The main predictor for the use of antibiotics was the presence of fever, and the presence isolated symptoms of infection was more decisive at the time of starting antibiotic treatment, rather than the combination of several symptoms fulfilling modified CDC criteria. In addition, just SAP diagnosed according to modified CDC criteria, and not pneumonia diagnosed by clinical criteria, proved to be an independent predictor of poor functional outcome three months after stroke (OR=4.84 (1.00-23.42)). In parallel, high-resolution chest CT images to confirm the diagnosis of SAP were obtained in 100 patients with severe strokes. Currently, these images are in the process of reading, and their usefulness for SAP management will be evaluated soon.

3. Relevance for future implications

The final results of the present project might be translated in the future into different clinical indications, at least in three different issues:

First, from a clinical point of view, we have identified a low adherence in current clinical practice to the modified CDC criteria for SAP diagnosis, both in terms of SAP diagnosis,

as well as for treatment indications, in terms of antibiotic initiation. We have also shown that pneumonia diagnosed according to modified CDC criteria represents an independent predictor of poor functional outcome after stroke, while pneumonia diagnosed according to clinical criteria, not fulfilling modified CDC criteria, does not worsen clinical outcome. From these data, we can suggest a systematic use of these criteria in clinical practice, both for SAP diagnosis and for antibiotic initiation, which might result in reduced antibiotic use, reducing therefore bacterial resistances, without compromising clinical outcome.

Second, we have identified a panel of blood biomarkers, including CRP, SAA and MR-proADM, able to predict SAP with high levels of sensitivity, specificity and accuracy. Furthermore, these biomarkers are commonly measured in routine clinical laboratories and therefore, the use of this panel in clinical practice could be immediate once the clinical indication is clearly defined. In a first scenario, determined by the high sensitivity of the panel, these biomarkers could be measured in patients with some symptoms of pneumonia (such as fever, which was defined as the covariate with the most importance for starting antibiotics). Negative results would allow antibiotic saving. In a second plausible scenario, determined by the high specificity of the panel, we would be able to identify a subgroup of patients at a very high risk of pneumonia, which would be the best candidate group to test future immunomodulatory drugs. Finally, although still far from an immediate clinical application, the new biomarkers identified in this project, such as YKL-40 and S100A12, open new research lines, not just as new biomarkers to enrich the previous panel, but also as new therapeutic targets against the development of SAP, with the aim of modulating stroke-induced immunosuppression. In future research projects we will go deeper into the omics data obtained in this project, with the aim of identifying new immunomodulatory therapies by systems biology, which could constitute an alternative to the use of antibiotics for the prevention of this dramatic complication.

As a future research line, we can envision a clinical trial based on the biomarkers' results. The biomarkers panel would be evaluated in patients with suspected infection (defined by the presence of an item of the CDC criteria, but not meeting them all), initiating antibiotics or not depending of the results of the panel. As a continuation of this project, we have obtained additional funding to carry out a new project, "Evaluating Lung Injury as a TargEt against Stroke-Associated Pneumonia (ELITE-

SAP)", from Instituto de Salud Carlos III. With this project, we will continue the clinical and preclinical studies in SAP during the proper years.

4. Bibliography

The present project has generated so far a total of nine presentations in national and international conferences, three publications in international journals, two more under review, and two doctoral theses.

CONFERENCE PRESENTATIONS

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DOCTORAL THESES

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