Loss of Braking Signals During Inflammation

A Factor Affecting the Development and Disease Course of Multiple Sclerosis

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Background: In a recent genome-wide transcriptional analysis, we identified a gene signature for multiple sclerosis (MS), which reverted back to normal during pregnancy. Reversion was particularly evident for 7 genes: SOCS2, TNFAIP3, NR4A2, CXCR4, POLR2J, FAM49B, and STAG3L1, most of which encode negative regulators of inflammation.

Objectives: To corroborate dysregulation of genes, to evaluate the prognostic value of genes, and to study modulation of genes during different treatments.

Design: Comparison study.

Setting: Italian referral center for MS.

Patients: Quantitative polymerase chain reaction measurements were performed for 274 patients with MS and 60 healthy controls. Of the 274 patients with MS, 113 were treatment-naïve patients in the initial stages of their disorder who were followed up in real-world clinical settings and categorized on the basis of disease course. The remaining 161 patients with MS received disease-modifying therapies (55 patients were treated with interferon beta, 52 with glatiramer acetate, and 54 with natalizumab) for a mean (SD) of 12 (2) months.

Conclusions: We demonstrate that there is a new molecular pathogenic mechanism that underlies the initiation and progression of MS. Defects in negative-feedback loops of inflammation lead to an overactivation of the immune system so as to predispose the brain to inflammation-sensitive MS.

Main Outcome Measures: Gene expression levels, relapse rate, and change in Expanded Disability Status Scale.

Results: We found a dysregulated gene pathway (P ≤ .006), with a downregulation of genes encoding negative regulators. The SOCS2, NR4A2, and TNFAIP3 genes were inversely correlated with both relapse rate (P ≤ .002) and change in Expanded Disability Status Scale (P ≤ .005). SOCS2 was modulated by both interferon beta and glatiramer acetate, TNFAIP3 was modulated by glatiramer acetate, and NR4A2 was not altered at all. No changes were induced by natalizumab.

To investigate the mechanisms behind the regulation of inflammation in multiple sclerosis (MS), we recently studied a physiological transitory condition (ie, pregnancy) in which the immune system strengthens its anti-inflammatory component and the disease frequently goes into remission. In particular, we conducted a genome-wide transcriptional analysis of peripheral blood mononuclear cells (PBMCs) obtained from women with MS and healthy women before, during, and after gestation. We identified 7 genes whose expression was altered in nonpregnant patients, and whose expression reverted to “normal” during gestation. The 7 genes were as follows: suppressor of cytokine signaling 2 (SOCS2); tumor necrosis factor, α-induced protein 3 (TNFAIP3); nuclear receptor subfamily 4, group A, member 2 (NR4A2); chemokine (C-X-C motif) receptor 4 (CXCR4); the polymerase (RNA) II (DNA directed) polypeptide J, 13.3kDa (POLR2J); family with sequence similarity 49, member B (FAM49B); and stromal antigen 3-like 1 (STAG3L1).

All genes, with the exception of STAG3L1, were previously reported to be associated with autoimmune diseases and are involved in the regulation of inflammatory processes. Even considering that many genes are involved in inflammatory responses and that 3 of the 7 genes do not have a clearly
defined role in inflammation (POLR2J encodes a subunit of RNA polymerase II, whereas FAM49B and STAG3L1 encode molecules of yet unknown function), it is impressive that the remaining genes encode anti-inflammatory effectors, being indeed downregulated in patients with MS. TNFAIP3 is critical in terminating tumor necrosis factor–induced NF-κB responses.10-12 NR4A2 functions as an inhibitor of inflammatory gene expression,13 SOCS2 inhibits signal transduction induced by cytokines,14 and CXCR4 regulates the trafficking of T-regulatory cells.15 Thus, data suggest that, besides the known molecular mechanisms underlying overactive inflammation in MS, there might be an alteration in mechanisms that act to counter-regulate or resolve inflammatory responses. Accordingly, similar mechanisms were identified as relevant to the pathology of other neurodegenerative diseases.16 On the other hand, although inducers of inflammation may be generated in a disease-specific manner, there is evidence for a remarkable convergence in the mechanisms responsible for the sensing and amplification of inflammatory processes that result in the production of neurotoxic mediators.16

In our study, we first aimed at corroborating, in different cohorts of subjects, our finding that SOCS2, TNFAIP3, NR4A2, CXCR4, POLR2J, FAM49B, and STAG3L1 are dysregulated in patients with MS. To overcome possible epistate-porphery phenomena,17,18 we analyzed an extremely homogeneous cohort of untreated patients with MS in the initial stages of their disorder. Furthermore, because our previous gene expression profiles were analyzed in a female cohort only, we checked for possible sex-specific differences by comparing gene expression profiles in men and women.

Our second aim was to evaluate the possible prognostic value of the 7 genes for the clinical evolution of MS. Hence, we analyzed the relationship between gene expression and clinical outcome, with respect to disability progression and relapse rates (RRs). Finally, we compared the specific immunoregulatory gene pathway with the targeted immunomodulation that appears to be associated with some differentially acting disease-modifying therapies (DMTs), such as interferon beta, glatiramer acetate, and natalizumab.

### METHODS

**PARTICIPANTS**

Our study was approved by our ethical committee of our hospital and conducted according to the Declaration of Helsinki. Informed consent was obtained from each individual. A total of 274 patients (199 women and 75 men) with definite relapsing-remitting MS were enrolled. Of these 274 patients, 113 (87 women and 26 men) had never been treated with DMT before being enrolled, and 161 (112 women and 49 men) had been treated with DMT. Of the 161 patients who had had DMT, 35 were treated with interferon beta (20 patients with Avonex [Biogen-Idec, Cambridge, Massachusetts]; 18 patients with Rebif [Merck-Serono, Geneva, Switzerland]; and 17 patients with Betaferon [Bayer Schering, Berlin, Germany]), 54 were treated with natalizumab (Tysabri [Biogen-Idec, Cambridge, Massachusetts]), and 52 were treated with glatiramer acetate (Copaxone; sanofi-aventis, Paris, France). Patients were treated for a mean (SD) duration of 12 (2) months (Table 1).

Patients treated with interferon beta were either positive (n = 20) or negative (n = 35) for anti-interferon beta neutralizing antibodies (NAb), whereas all natalizumab-treated patients were negative for antinatalizumab NAb.19,20 Sixty age-matched healthy individuals (39 women and 21 men) were included as controls.

**CLINICAL FOLLOW-UP AFTER BASELINE EVALUATION OF GENE EXPRESSION**

Baseline samples were obtained from the treatment-naive cohort during a 4-year period (ie, from October 2004 to November 2008). After blood samples were obtained, patients were clinically followed up in our MS center, for at least 2 years and in real-world clinical settings. For routine clinical monitoring, subjects were required to visit the clinic for a baseline evaluation and, every 3 months thereafter, for an evaluation of blood parameters and to determine whether there were any adverse effects. Neurologic examinations, with classification using the Expanded Disability Status Scale (EDSS) and the recording of relapses, were performed every 6 months or in case of need. A relapse was defined as the appearance of a new symptom or a worsening of a preexisting symptom, lasting more than 24 hours and producing a modification in the functional system of the EDSS.21 All relapses were treated with high-dose methylprednisolone hemisuccinate sodium (15 mg/kg for 10 days).

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Treatment-Naive Patients</th>
<th>NAT-Treated Patients</th>
<th>GA-Treated Patients</th>
<th>IFN-β-Treated Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample size, No.</td>
<td>113</td>
<td>54</td>
<td>52</td>
<td>20</td>
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<tr>
<td>Sex, No.</td>
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<td>41</td>
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<tr>
<td>M</td>
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<td>13</td>
<td>16</td>
<td>9</td>
</tr>
<tr>
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<td>54/0</td>
<td>52/0</td>
<td>20/0</td>
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<tr>
<td>Age at start of therapy, y, median (range)</td>
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<td>34 (18-55)</td>
<td>38 (13-58)</td>
<td>36 (24-59)</td>
</tr>
<tr>
<td>Disease duration at start of therapy, mo, median (range)</td>
<td>17 (2-39)</td>
<td>108 (4-363)</td>
<td>68 (4-359)</td>
<td>27 (1-139)</td>
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<tr>
<td>EDSS score at start of therapy, median (range)</td>
<td>1.0 (0-2.0)</td>
<td>3.5 (1.5-7.0)</td>
<td>1.5 (0-5.5)</td>
<td>1.5 (0-3.0)</td>
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<tr>
<td>RR 2 y before therapy, median (range)</td>
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<td>2.5 (2-6)</td>
<td>1.3 (0-3)</td>
<td>1.2 (0-2)</td>
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<tr>
<td>EDSS score 1 y after therapy, median (range)</td>
<td>3.0 (1.0-7.0)</td>
<td>1.5 (0-5.5)</td>
<td>1.5 (0-3.0)</td>
<td>1.0 (0-3.5)</td>
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<tr>
<td>RR 1 y after therapy, median (range)</td>
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<td>0.2 (0-2)</td>
<td>0.2 (0-2)</td>
<td>0.1 (0-1)</td>
</tr>
</tbody>
</table>

Table 1. Demographic and Clinical Characteristics of Patients at Baseline and During Therapy

Abbreviations: CIS, clinically isolated syndrome; EDSS, Extended Disability Status Scale; GA, glatiramer acetate; IFN-β, interferon beta; NAb, neutralizing antibodies; NAT, natalizumab; RR, relapse rate; RRMS, relapsing-remitting multiple sclerosis.
After confirmed diagnosis of relapsing-remitting MS, all patients received first-line DMT (ie, 100 patients received interferon beta, and 13 patients received glatiramer acetate). At the end of the follow-up (ie, November 2010), there were 73 patients (64.6%) still being treated with either interferon beta or glatiramer acetate, whereas 8 patients (7.1%) discontinued treatment because they wanted to become pregnant. As a whole, these 81 patients (71.7%) were considered to have a “nonaggressive” form of the disease. On the contrary, 20 patients (17.7%) were categorized as having first-line DMT that failed completely, because they had experienced at least 2 relapses during the prior year. These patients were switched to second-line treatments, such as natalizumab (n = 17) or mitoxantrone hydrochloride (n = 3), and were considered to have an “aggressive” form of the disease (Figure 1).

First-line DMT had also failed in a second group of 8 patients (7.1%), mostly because of adverse events; these patients were switched to alternative treatments such as methotrexate sodium (n = 1) or azathioprine sodium (n = 7). Also, it was determined during follow-up that 4 patients (3.5%) had developed secondary progressive MS. These 12 patients were excluded from the present analysis owing to the possible different phenotypes and the paucity of subjects in these series.

### RNA EXTRACTION AND MESSENGER RNA QUANTIFICATION

Whole blood samples, collected into a Tempus vacuette, were extracted using the ABI Prism 6100 Nucleic Acid PrepStation (Applied Biosystems, Foster City, California), following the manufacturer’s instructions. Total RNA (final concentration, 5 ng/µL) was reverse-transcribed using random hexamer primers. Complementary DNA was then used as a template for a blind real-time reverse transcription–polymerase chain reaction analysis based on a 5′-nuclease assay. Expression of CXCR4, TNFAIP3, SOCS2, NR4A2, FAM49B, POLR2J, and STAG3L1 was analyzed using Applied Biosystems' TaqMan gene expression products. Relative expression levels of targets were calculated by the comparative cycle threshold method and by normalizing to glyceraldehyde-phosphate-dehydrogenase.

### STATISTICAL ANALYSIS

Clinical analyses were performed in which we calculated an RR, defined as the number of confirmed relapses per patient-year, and a rate of disease progression measured by the mean change from baseline in the EDSS score per year (ΔEDSS). All statistical analyses were performed with GraphPad PRISM version 4.00 (San Diego, California). Data were evaluated by means of the Kruskal-Wallis test and the Mann-Whitney U test. Nonlinear regression analysis was conducted to determine the relation between messenger RNA (mRNA) levels and both RR and ΔEDSS. All reported P values were based on 2-tailed statistical tests, with an α level of .05 indicating the threshold of statistical significance.

### RESULTS

#### UNTREATED PATIENTS WITH MS AND HEALTHY CONTROLS

Deregulated gene expression was observed in untreated patients with MS compared with healthy controls (P ≤ .007). In particular, a lower mRNA expression of CXCR4, SOCS2, TNFAIP3, and NR4A2 was observed in untreated patients with MS (P < .001), whereas a higher mRNA expression of FAM49B, POLR2J, and STAG3L1 was observed in healthy controls (P ≤ .007) (Figure 2). To account for potential sex-related differences in gene expression, we also compared mRNA levels in men and women: sex-related differences did not reach statistical significance, considering both untreated patients with MS (P ≥ .08) and healthy controls (P ≥ .16).

### RELATIONSHIP BETWEEN GENE EXPRESSION AND CLINICAL OUTCOME AT FOLLOW-UP

First, we confirmed the definition of “aggressive” and “nonaggressive” for delineating different clinical courses in patients; both the RR and ΔEDSS were higher in patients who were switched to natalizumab or mitoxantrone therapy, compared with patients who were still treated with interferon beta or glatiramer acetate and patients who wanted to become pregnant (P ≤ .004) (Table 2). To examine whether the dysregulated expression of the genes was associated with a worse clinical course, we compared mRNA levels in aggressive and nonaggressive clinical courses in patients. The comparison showed that the levels of gene expression for SOCS2 (P = .04),
NR4A2 ($P = .003$), and TNFAIP3 ($P = .001$) were lower in patients who had an aggressive course compared with patients who had a nonaggressive course. On the contrary, the level of gene expression for POLR2J was higher in patients who had an aggressive course compared with patients who had a nonaggressive course ($P = .05$). On the
other hand, the levels of gene expression for FAM49B approached statistical significance (P = .06), whereas no differences in gene expression for CXCR4 and STAG3L1 were detected in the 2 groups of patients (Figure 3). Afterward, we examined the relationship between baseline mRNA levels and both RR and ∆EDSS. Interestingly, the levels of gene expression for SOCS2, NR4A2, and TNFAIP3 were inversely correlated with both RR and ∆EDSS. However, variables needed to be log-transformed because the relationship between untransformed variables appeared nonlinear. After log transformation of variables, the correlation coefficient for ∆EDSS was −0.423 (P = .005) when related to SOCS2 expression, −0.597 (P < .001) when related to NR4A2 expression, and −0.618 (P < .001) when related to TNFAIP3 expression (Figure 4). Likewise, correlation coefficients for RR were −0.392 (P = .002), −0.517 (P < .001), and −0.586 (P < .001), respectively (Figure 5).

PATIENTS TREATED WITH INTERFERON BETA

Neutralizing antibodies are capable of abrogating both the biological and clinical action of interferon beta. Hence, to study the effect of interferon beta on gene expression, we performed analyses on 2 groups of interferon beta–treated patients: NAb-positive and NAb-negative patients.

Following interferon beta treatment, in NAb-negative patients, 3 of 7 genes (ie, SOCS2, CXCR4, and FAM49B) reverted to expression levels displayed by healthy controls (P = .20). This was not the case for the remaining genes, which had levels of expression that did not change during therapy. By contrast, in NAb-positive patients, expression was comparable to levels detected in untreated patients with MS (P = .04) (Figure 2). No differences in gene expression were observed between men and women in both groups.

PATIENTS TREATED WITH GLATIRAMER ACETATE

In patients treated with glatiramer acetate, the level of expression of 5 of 7 genes was shown to be affected by therapy. In particular, TNFAIP3, SOCS2, FAM49B, POLR2J, and STAG3L1 reverted their expression to levels displayed by healthy controls (P = .06), whereas glatiramer acetate did not affect either NR4A2 or CXCR4 expression (Figure 2). No differences in gene expression were observed between men and women.

PATIENTS TREATED WITH NATALIZUMAB

In patients treated with natalizumab, 1 of 7 genes (ie, FAM49B) reverted its expression to normal (P = .001); these natalizumab-treated patients maintained the same levels of CXCR4, POLR2J, NR4A2, STAG3L1, and SOCS2 compared with untreated patients (Figure 2). On the contrary, despite the initial downregulation of TNFAIP3, the gene was further downregulated in patients who received natalizumab therapy (P = .004) compared with untreated patients (Figure 2C). No significant differences were observed between men and women.

COMMENT

In a recent genome-wide transcription analysis comparing PBMCs from patients with MS and healthy controls before, during, and after pregnancy, we have identified 7 genes (ie, CXCR4, SOCS2, TNFAIP3, NR4A2, FAM49B, STAG3L1 and POLR2J) whose expression was dysregulated before gestation and reverted by the pregnancy process. Of these 7 genes, 3 (ie, POLR2J, FAM49B, and STAG3L1) do not have a clearly defined role in inflammation, whereas 4 genes (ie, CXCR4, SOCS2, TNFAIP3,
and NR4A2) encode negative regulators of inflammatory responses.\textsuperscript{10-15,28} Previous studies had already shown a consistent association between autoimmune diseases (e.g., rheumatoid arthritis, systemic lupus erythematosus, and ankylosing spondylitis) and the latter 4 genes.\textsuperscript{5-9} Hence, these genes have been proposed to be critical “braking” molecules, acting to control inflammation in autoimmunity. Accordingly, in our previous study,\textsuperscript{4} we had observed markedly decreased levels of CXCR4, SOCS2, TNFAIP3, and NR4A2 expression in patients with MS, which might contribute to the chronic invasive immune processes in these patients, resulting from an initial aberrant inflammation. The obvious rationale for such an observation is that, in patients with MS (and probably

Figure 3. Comparison of mean gene expression levels of (A) CXCR4, (B) SOCS2, (C) TNFAIP3, (D) NR4A2, (E) FAM49B, (F) POLR5J, and (G) STAG3L1 in healthy controls and in patients with multiple sclerosis who were subdivided into “aggressive” and “nonaggressive” on the basis of their clinical course after providing a blood sample. Horizontal bars indicate median values.
in patients with autoimmune diseases overall), there is a dysregulation of molecular mechanisms underlying anti-inflammatory phenotypes, leading to an overactivation of the immune system.

As a whole, the results of our present study, analyzing different sets of treatment-naive patients with MS and healthy controls, confirm this specific gene dysregulation in MS. Given the immunological variables in MS, it is significant that the present results stem from the analysis of an extremely homogeneous cohort of patients in the initial stages of MS. Indeed, all of the patients were in the initial stages of their disorder, and their levels of neurological disability were still low. Therefore, patients were at a specific stage of the disease at which inflammation is still highly prevalent and the MS immune response more homogenous. This strict selection process allowed us to conclude that the altered expression of negative regulators of inflammation is a central pathogenic mechanism and not only a consequence of the disease progression. On the other hand, this finding agrees with the finding of a silent MS trait that is associated with suppressed expression of critical negative-feedback effectors such as nuclear receptors (including NR4A2), which regulate induction and resolution of inflammatory responses, specifically through trans repression mechanisms in macrophages and microglia.

Although men and women have genetically identical autosomal chromosomes, they could result in sex-

**Figure 4.** Relationships between annualized changes in Expanded Disability Status Scale score ($\Delta$EDSS) and baseline messenger RNA levels of SOCS2 (A and B), NR4A2 (C and D), and TNFAIP3 (E and F) in 101 patients with multiple sclerosis. B, D, and F, Correlations between $\Delta$EDSS and gene expression after log transformation of nonnormally distributed variables. $P=0.005$ for all correlations.
specific transcriptional regulation leading to differential mRNA products for some genes. Because, in our previous study, we analyzed gene expression profiles in women only, in our present study, we also evaluated possible sex-related differences, comparing gene expression patterns between men and women. Although most of the target genes have been reported to be regulated by estrogens, we did not find evidence of sex-related differences in gene expression.

Because detection of differentially regulated genes may lead to the identification of new therapeutic targets, we next asked whether our gene expression findings could have any clinical relevance for patients with MS. To this end, we have further analyzed the gene expression profiles of 113 treatment-naive patients, who, after providing blood samples, were followed up in real-world clinical settings. In particular, we examined whether there was a relationship between baseline expression and disease course, as measured by RR and disease progression. Clearly, operational criteria were proposed for differentiating clinical courses into aggressive and nonaggressive. Nevertheless, we believe that the group of patients defined as having an aggressive disease course is actually characterized by a different phenotype of MS. In fact, these patients have experienced at least 2 relapses in the prior 12 months, despite their having received immunomodulating treatment. This worse disease course is likely due to pathogenic mechanisms underlying a par-

Figure 5. Relationships between relapse rate (RR) and baseline messenger RNA levels of SOCS2 (A and B), NR4A2 (C and D), and TNFAIP3 (E and F) in 101 patients with multiple sclerosis. B, D, and F, Correlations between RR and gene expression after log transformation of nonnormally distributed variables. P<.002 for all correlations.
particularly strong inflammation that is not responding to both interferon beta and glatiramer acetate. Accordingly, a recent study\textsuperscript{34} found an enhanced type 1 interferon signature in monocytes from interferon beta–treated nonresponsive patients who also had a more aggressive disease course.

As a whole, the results of our present study show that altered gene expression correlates with clinical features. In particular, our findings indicate a correlation between reduced expression of SOCS2, NR4A2, and TNFAIP3 and a worse disease course. Baseline levels were indeed lower in patients with a more malignant disease course than in patients with a more benign disease course, and also negatively correlated with both RR and ΔEDSS. Given this clinical correlation, it is important to note the key role played by those genes in critical negative-feedback loops of inflammation. SOCS2 is a well-known negative regulator of cytokines that signal through the Jak-STAT pathway.\textsuperscript{14} Likewise, both TNFAIP3 and NR4A2 are part of negative-feedback loops that mediate their inhibitory function by downregulating proinflammatory signaling pathways, including those controlling NF-κB\textsuperscript{39,12} and IRF3-dependent gene expression.\textsuperscript{13} NR4A2 also functions as an inhibitor of neurotoxic gene expression in microglia and astrocytes via a CoREST-dependent transrepression pathway.\textsuperscript{13,16,31} Taken together, these findings corroborate our initial statement that MS arises from a dysregulation of “braking signals” in inflammation, rather than merely from an overactive proinflammatory reaction. This observation offers a clear model by which to consider the clinical data presented here: with lower SOCS2, NR4A2, and TNFAIP3 gene expression levels, one can expect worse inflammation and thus a worse clinical course.

A third aim of our study was to compare gene expression with the immunomodulation that appears to be associated with common DMTs (namely, interferon beta, glatiramer acetate, and natalizumab). With respect to the interferon beta–treated patients, we observed a modulation of 3 genes (ie, CXCR4, SOCS2, and FAM49B) that reverted to expression levels displayed by healthy controls. Evidence of an actual effect of interferon beta is given by previous studies that have already shown controls. Evidence of an actual effect of interferon beta is given by previous studies that have already shown.

Our findings clearly show that there are partial differential overlapping mechanisms of protection between DMT and pregnancy, because each of the drugs leads to the reversion in expression of some, but not all, of the pregnancy-related genes. The importance of modulating those genes to obtain a clinical improvement is underlined by our previous observation that women with MS who relapse during pregnancy have a delayed modulation of gene expression compared with relapse-free women.

In conclusion, our findings suggest that there is a new molecular pathogenic mechanism that underlies the initiation and progression of MS. Defects in the negative regulation of inflammatory responses lead to an overactivation of the immune system so as to predispose certain organ systems to inflammation-sensitive pathologies such as MS. All this information can be used to design powerful new diagnostic tools and can also be used to identify new therapeutic targets.

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