Patients with multiple sclerosis (MS) suffer with age an early immunosenescence process, which influence the treatment response and increase the risk of infections. We explored whether lipid-specific oligoclonal IgM bands (LS-OCMB) associated with highly inflammatory MS modify the immunological profile induced by age in MS. This cross-sectional study included 263 MS patients who were classified according to the presence (M+, n=72) and absence (M-, n=191) of LS-OCMB. CSF cellular subsets and molecules implicated in immunosenescence were explored. In M+ patients, aging induced remarkable decreases in absolute CSF counts of CD4+ and CD8+ T lymphocytes, including Th1 and Th17 cells, and of B cells, including those secreting TNF-alpha. It also increased serum anti-CMV IgG antibody titers (indicative of immunosenescence) and CSF CHI3L1 levels (related to astrocyte activation). In contrast, M+ patients showed an age-associated increase of TIM-3 (a biomarker of T cell exhaustion) and increased values of CHI3L1, independently of age. Finally, in both groups, age induced an increase in CSF levels of PD-L1 (an inductor of T cell tolerance) and activin A (part of the senescence-associated secretome and related to inflamming). These changes were independent of the disease duration. Finally, this resulted in augmented disability. In summary, all MS
patients experience with age a modest induction of T-cell tolerance and an activation of the innate immunity, resulting in increased disability. Additionally, M- patients show clear decreases in CSF lymphocyte numbers, which could increase the risk of infections. Thus, age and immunological status are important for tailoring effective therapies in MS.

**Keywords:** multiple sclerosis, aging, innate immunity, adaptive immunity, inflammation

**INTRODUCTION**

Multiple sclerosis (MS) is an autoimmune disease that is characterized by demyelination, chronic inflammation, and neuronal loss, causing irreversible damage to the central nervous system (CNS). Accumulating evidence suggests that aging is a risk factor for the progression of MS, and late onset of the disease (40-50 years of age) is associated with earlier conversion to progressive phases (1). Additionally, age reduces the capability of the CNS to remyelinate (2) and increases extracellular accumulation of iron (3), mitochondrial dysfunction (4), and chronic microglia activation (5).

Age-related changes in the immune system have been extensively studied (6, 7). Aging is generally considered to be associated with chronic low-grade inflammation, which affects the innate and adaptive immune systems in a phenomenon known as “immunosenescence” (8). The innate immune system increases the secretion of pro-inflammatory cytokines and proteases, including Tumor Necrosis Factor (TNF)-α, interleukin (IL)-6, and metalloproteases. This is associated with reduced tissue clearance and phagocytosis capacity by myeloid cells (9). On the other hand, the adaptive immune system displays a reduction in the number of naïve T cells due to thymic involution, which reduces their ability to react against new antigens (10).

Importantly, the number of memory T cells in the bloodstream increases, especially the CD8 subset (11). In particular, an increase in memory T cells and antibodies specific for cytomegalovirus (CMV) has been reported, which is broadly considered to be a hallmark of immunosenescence (12). Remarkably, this process that usually occurs in people older than 65 years arises about 20 years earlier in different inflammatory diseases including MS. This phenomenon is named as premature or early immunosenescence (13–15).

Investigating the influence of a highly inflammatory disease in the immunosenescence process could help to identify whether age-related changes are a uniform process in MS or depend on patient idiosyncrasies.

To explore this, we studied the effect of aging in the adaptive and innate immune responses in the CSF of MS patients. We also explored whether the intrathecal synthesis of lipid-specific oligoclonal IgM bands (LS-OCMB), a well-established marker of a high inflammatory disease course in MS (16, 17), plays a role in this process. Understanding the age-related alterations in the immune system of MS patients is critical for the development of targeted therapeutic approaches and the discovery of novel potential markers of the progression of the disease.

**MATERIALS AND METHODS**

**Study Approval**

This study was approved by the Ethical Committee of Ramón y Cajal University Hospital (Madrid, Spain). Written informed consent was obtained from every patient before inclusion in the study.

**Patients**

In this cross-sectional prospective study, we included 263 consecutive patients (149 females/114 males) who were diagnosed with MS at Ramón y Cajal University Hospital (Madrid, Spain) according to modified McDonald criteria (18). Patients did not receive any disease-modifying treatment before inclusion. Patient characteristics are shown in Table 1. The expanded disability status scale (EDSS) score and the MS severity score (MSSS) were evaluated at lumbar puncture, or in case the patient was in a relapse at that moment, one month after when the clinical situation was stabilized. EDSS score was measured in the whole patient cohort, MSSS in the 161 patients with more than six months of disease duration at lumbar puncture (41 M+ and 120 M-), and the number of relapses in the first year in the 119 patients with RRMS and more than six months of disease duration (34 M+ and 101 M-).

**Samples**

Paired serum and CSF samples were always obtained for clinical purposes. Fresh CSF samples were centrifuged at 500 g for 10 min. The cellular pellet was resuspended and analyzed for subsequent flow cytometry studies as described below. After centrifugation, CSF and serum samples were aliquoted and stored at -80°C until assessment.

**Immunoglobulin G and M Oligoclonal Band Detection**

Serum and CFS IgG, IgM, and albumin were quantified by nephelometry in an Immage nephelometer (Beckman Coulter, Brea, CA). Oligoclonal IgG and IgM bands were studied in paired CSF and serum samples by isoelectric focusing and immunoblotting as described previously (16, 19). The presence of intrathecal IgG or IgM synthesis was demonstrated by the appearance of two or more oligoclonal bands in CSF, not present in paired serum sample. Lipid specific IgM bands were assessed in the CSF of patients showing intrathecal IgM synthesis as previously described (16). Briefly, oligoclonal IgM bands are separated by isoelectrofocusing and transferred to nitrocellulose membranes coated with different lipids and blocked with Polypep (Merck). A membrane blocked with polypep is used as negative control. The presence of anti-lipid IgM antibodies restricted to the CSF is then evidenced by
immunoblotting with anti-human IgM antibodies labeled with biotin and with streptavidin labeled with alkaline phosphatase. A representative image of anti-lipid IgM bands is shown in Supplementary Figure 1.

CSF Leukocyte Subpopulations

The following monoclonal antibodies were used in the study: CD14-FITC, IFN-γ-FITC, GM-CSF-PE, CD3-PerCP, TNF-α-PerCP-Cy5.5, CD16-PE-Cy7, CD19-PE-Cy7, CD56-APC, CD8-APC-H7, CD3-BV421, and CD45-V500 (BD Biosciences, San Diego, CA). IL-17-APC was obtained from R&D Systems, Minneapolis, MN. For surface antigen identification, cellular pellets were incubated for 4 hours at 37°C in 5% CO2 with 50 ng/ml Phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich, St. Louis, MO) and 750 ng/ml Ionomycin (Sigma-Aldrich) in presence of 2 µg/ml Brefeldin A (GojiPlugs, BD Biosciences) and 2.1 µM Monensin (Goji Stop, BD Biosciences). After incubation, cells were washed and stained with the monoclonal antibodies recognizing the surface antigens. Then, cells were washed and analyzed in a FACSCanto II flow cytometer (BD Biosciences). Data analysis was performed using the software FACSDiva V.8.0 (BD Biosciences) and the gating strategy shown in Supplementary Figures 2, 3. All labeled cells were acquired to calculate total cell numbers.

Flow Cytometry Analysis

For surface antigen identification, cellular pellets obtained after CSF centrifugation were resuspended in the residual volume (about 100 µl), stained with the appropriate amounts of monoclonal antibodies for 30 minutes at 4°C in the dark, washed twice with PBS, and analyzed in a FACSCanto II flow cytometer (BD Biosciences). For intracellular cytokine detection, cellular pellets were stimulated and stained for flow cytometry analysis as described previously (20). In brief, cellular pellets were incubated for 4 hours at 37°C in 5% CO2 with 50 ng/ml Phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich, St. Louis, MO) and 750 ng/ml Ionomycin (Sigma-Aldrich) in presence of 2 µg/ml Brefeldin A (GojiPlugs, BD Biosciences) and 2.1 µM Monensin (Goji Stop, BD Biosciences). After incubation, cells were washed and stained with the monoclonal antibodies recognizing the surface antigens. Then, cells were washed, fixed and permeabilized with Cytofix/Cytoperm Kit (BD Biosciences), washed twice and subjected to intracellular staining with monoclonal antibodies recognizing different cytokines. Then, cells were washed and analyzed in a FACSCanto II flow cytometer. Data analysis was performed using the software FACSDiva V.8.0 (BD Biosciences) and the gating strategy shown in Supplementary Figures 2, 3. All labeled cells were acquired to calculate total cell numbers.

Detection of Soluble Molecules in CSF

We used ELISA to explore the CSF values of the following molecules: activin A (R&D Systems, MN), chitinase 3-like 1 (CHI3L1; Quidel Corporation, San Diego, CA), C3 complement component (Abcam; Cambridge, UK), neurofilament light chains (NfL; Uman Diagnostics, Sweden), programmed death-ligand 1 (PD-L1; R&D Systems), and T-cell immunoglobulin and mucin domain 3 (TIM-3; Bio-Technne, R&D Systems). All assays were run according to the manufacturer’s instructions with the exception of NfL, for which 10 and 50 µl of CSF were assayed for every patient.

Anti-CMV IgG ELISA

Serum anti-CMV IgG antibodies were measured by ELISA (Zeus Scientific, USA) according to the manufacturer’s instructions. The results were expressed as an index value (IV) that was calculated as follows: 10 x sample absorbance/cut-off value. Samples were analyzed in duplicate for each test.

Statistical Analyses

Statistical analyses were done using the software GraphPad Prism 6.0 (GraphPad Prism Inc., La Jolla, CA) and Stata 16 (StataCorp LLC, Lakeway, TX). For continuous variables, we used the Mann-Whitney U-test with Bonferroni post-hoc correction or the Kruskal-Wallis test with Dunn’s multiple comparison post-test when comparing 3 or more groups. The chi-squared test was used to compare categorical variables. P-values below 0.05 were considered as significant. Spearman correlation was used to test for associations between groups, and the Spearman r and p values are reported in each instance. Multivariate regression

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**TABLE 1 | Clinical and demographic data of patients included in the study.**

|                         | Total patients (n=263) | M- patients (n=191) | M+ patients (n=72) | P        |
|-------------------------|------------------------|---------------------|-------------------|----------|
| Age (years), Median (range) | 39.0 (16-65)           | 40.0 (16-65)        | 35.0 (18-62)      | ns       |
| Disease duration (months) | 7.4 (3.2-32.1)         | 8.4 (3.7-45.4)      | 6.5 (2.0-15.5)    | ns       |
| Sex (male/female)        | 114/139                | 89/111              | 34/38             | ns       |
| Disease onset (FF/FP)    | 231/32                 | 171/20              | 60/12             | ns       |
| EDSS score (*)           | 1.5 (1.5-2.0)          | 1.5 (1.5-2.0)       | 2.0 (1.5-3.0)     | 0.004    |
| MSSS score (*)           | 4.3 (2.4-5.0)          | 4.3 (2.4-5.2)       | 5.9 (4.3-7.9)     | <0.0001  |
| N. of Relapses in the previous year (**) | 1 (1-2)               | 1 (1-1)             | 2 (1-2)          | <0.0001  |
| CSF CHI3L1 (ng/ml) (*)   | 209.5 (159.5-284.8)    | 200.0 (156.0-266.2) | 246.5 (183.1-395.7) | 0.017    |
| CSF NfL (pg/ml) (*)      | 994.4 (567.3-2058.7)   | 842.6 (466.0-1364.5)| 1558.9 (795.7-4063.1)| 0.0002   |
| CSF C3 (ng/ml) (*)       | 9586 (6808-13744)      | 8172 (6256-10856)   | 10498 (759.7-15151)| 0.018    |
| IgG Index (*)            | 0.16 (0.11-0.27)       | 0.14 (0.10-0.22)    | 0.24 (0.15-0.42)  | <0.0001  |
| IgM Index (*)            | 4.24 (3.10-5.65)       | 4.24 (3.03-5.43)    | 4.24 (3.36-5.89)  | ns       |
| Alb Index (*)            | 4.24 (3.10-5.65)       | 4.24 (3.03-5.43)    | 4.24 (3.36-5.89)  | ns       |

( ): Data shown as Median (25-75% IQR); (**) : Explored in patients with at least six months of disease duration. Alb, Albumin; CHI3L1, Chitinase 3-like 1; C3, C3 complement component; CSF, cerebrospinal fluid; EDSS, Expanded Disability Status Scale; IQR, Interquartile range; LP, lumbar puncture; M+ patients, Those showing lipid-specific oligoclonal IgM bands; M-, patients, Those lacking lipid-specific oligoclonal IgM bands; MSSS, Multiple Sclerosis Severity Score; N., number; NfL, neurofilament light chain; ns, not significant; PP, Progressive onset; RR, relapsing remitting onset. P values are referred to M+ vs M- patient comparisons. Continuous variables were analyzed using Mann-Whitney U test and categorical ones by Chi-square tests.
analysis was used to explore the relationship between age and immunological factors and between age and disability scores while adjusting for disease duration.

RESULTS

Patient Characteristics

We investigated the association between aging and intrathecal immune response in MS by studying a large cohort of treatment-naive MS patients (n=263). We stratified them in two groups according to the absence (M-, n=191) and presence (M+, n=72) of LS-OCMB. Clinical, demographic, and laboratory data are shown in Table 1. M+ patients showed higher number of relapses in the previous year (p<0.0001), and higher values of the EDSS (p=0.004) and the MSSS (p<0.0001) scores. In addition, they showed increased CSF values of CHI3L1 (p=0.017), neurofilament light chains (NfL) (p=0.0002), C3 complement factor (p=0.018) and of the IgM index (p<0.0001).

Aging Diminishes Numbers of Intrathecal Lymphocytes and NK Cells in M- Patients

Next, we studied age-related changes in the main leukocyte subsets in the CSF. The results are shown in Table 2. M- patients showed a remarkable decrease of mononuclear cell numbers (p<0.0001) due to a drop-in lymphocytes (p<0.0001) and natural killer cells (NK) (p=0.0001). A similar reduction was found in CD4+ (p<0.0001) and CD8+ (p<0.0001) T cells and in B cells (p<0.0001). In contrast, monocyte numbers did not show any significant changes (Table 2). We also explored the intracellular production of pro-inflammatory cytokines by CSF lymphocytes. In the M+ group, age induced significant decreases of CD4+ and CD8+ T cells producing IFN-γ, TNF-α, and GM-CSF, as well as CD4+ T cells producing IL-17. A reduction in B cells producing TNF-α was observed in both groups of patients (Table 2).

To rule out the effect of disease duration on these correlations, we performed a multivariable linear regression analysis between age and leukocyte numbers adjusting for disease duration. Most associations remained significant with the only exceptions of CD4+ T cells producing IL-17 in M- patients and B cells producing TNF-α in M+ patients that were lost (Figures 1A, B and Supplementary Table 1).

To investigate the age at which intrathecal leukocyte decline occurs, we classified patients according to their age in subgroups of five years (i.e., ≤25, 26-30, 31-35 years, and so on). The highest changes in lymphocyte numbers were observed in M- patients older than 45 years (Figure 2A). The same results were observed when studied CSF CD4+ and CD8+ T cell numbers (Figures 2B, C).

### Table 2 | Correlations between age and variables related to immune response or axonal damage.

| Variable | Total patients (n=263) | M- patients (n=191) | M+ patients (n=72) |
|----------|----------------------|-------------------|-------------------|
| Leukocyte subsets (cells/ml) | r | p | r | p | r | p |
| Total Mononuclear cells | -0.40 | <0.0001 | -0.46 | <0.0001 | 0.02 | 0.886 |
| Total Lymphocytes | -0.40 | <0.0001 | -0.47 | <0.0001 | 0.02 | 0.889 |
| CD4+ T cells | -0.37 | <0.0001 | -0.45 | <0.0001 | 0.07 | 0.597 |
| CD4+ T cells IFN-gamma+ | -0.31 | 0.0002 | -0.33 | 0.0007 | -0.15 | 0.364 |
| CD4+ T cells TNF-alpha+ | -0.32 | 0.0001 | -0.35 | 0.0003 | -0.14 | 0.411 |
| CD4+ T cells IL-17+ | -0.34 | <0.0001 | -0.31 | 0.0012 | -0.34 | 0.042 |
| CD4+ GM-CSF+ | -0.41 | 0.002 | -0.44 | 0.003 | -0.28 | 0.378 |
| CD8+ T cells | -0.34 | <0.0001 | -0.41 | <0.0001 | 0.02 | 0.852 |
| CD8+ T cells IFN-gamma+ | -0.30 | 0.0003 | -0.32 | 0.0006 | -0.11 | 0.510 |
| CD8+ T cells TNF-alpha+ | -0.27 | 0.0014 | -0.29 | 0.003 | -0.08 | 0.614 |
| CD8+ T cells IL-17+ | -0.21 | 0.0136 | -0.15 | 0.121 | -0.23 | 0.172 |
| CD8+ GM-CSF+ | -0.43 | 0.001 | -0.49 | 0.001 | -0.23 | 0.477 |
| CD19+ B cells | -0.41 | <0.0001 | -0.49 | <0.0001 | 0.02 | 0.847 |
| CD19+ TNF-alpha+ | -0.41 | 0.002 | -0.36 | 0.02 | -0.68 | 0.01 |
| CD19+ GM-CSF+ | -0.31 | 0.02 | -0.25 | 0.103 | -0.65 | 0.02 |
| Total NK cells | -0.31 | 0.0002 | -0.39 | 0.0001 | -0.08 | 0.590 |
| Total Monocytes | -0.09 | 0.142 | -0.13 | 0.070 | 0.03 | 0.795 |
| Soluble factors | | | | | | |
| PD-L1 (pg/ml) | 0.34 | <0.0001 | 0.30 | 0.0011 | 0.56 | 0.0005 |
| TIM-3 (pg/ml) | 0.30 | 0.0012 | 0.27 | 0.04 | 0.41 | 0.002 |
| IgG anti-CMV (IV) | 0.28 | 0.0003 | 0.31 | 0.0006 | 0.17 | 0.247 |
| CHI3L1 (ng/ml) | 0.41 | <0.0001 | 0.49 | <0.0001 | 0.29 | 0.057 |
| Activin A (pg/ml) | 0.46 | <0.0001 | 0.47 | <0.0001 | 0.44 | 0.003 |
| NfL (pg/ml) | -0.07 | 0.368 | 0.06 | 0.518 | -0.28 | 0.051 |
| C3 (mg/ml) | 0.18 | 0.098 | 0.18 | 0.220 | 0.27 | 0.085 |

C3, C3 complement component; CHI3L1, Chitinase 3-like 1; CMV, Cytomegalovirus; CSF, Cerebrospinal fluid; GM-CSF, Granulocyte/macrophage-colony stimulating factor; IV, Index Value; NK, Natural Killer; IFN, Interferon; PD-L1, Programmed Death-ligand 1; TIM-3, T-cell immunoglobulin and mucin domain-3; TNF, Tumor necrosis factor; IL, Interleukin; M+ patients, those showing lipid-specific oligoclonal IgM bands; M- patients, those lacking lipid-specific oligoclonal IgM bands. All variables were quantified in CSF with the exception of IgG anti-CMV, quantified in serum. r and p values were determined by Spearman correlation.
Remarkably, no differences were observed in the M+ group, even in patients older than 50 years.

In view of these results, we classified M+ and M- patients in two groups (Age \( \leq 45 \) or \( > 45 \) years) and further explored the influence of age in the numbers of CD4+ and CD8+ T cells producing IFN-gamma, TNF-alpha, and GM-CSF (Figure 3). M- patients older than 45 years showed lower CSF numbers of CD4 and CD8 T cells producing IFN-gamma (\( p=0.004 \) and \( p=0.003 \) respectively), TNF-alpha (\( p=0.002 \) and \( p=0.004 \) respectively), and GM-CSF (\( p=0.029 \) and 0.005 respectively).
FIGURE 2 | Changes in CSF lymphocyte counts in MS patients classified according to age and to the absence or presence of lipid-specific oligoclonal IgM bands (LS-OCMB). Absolute cell numbers (N.) of CSF total lymphocytes (A), CD4+ (B) and CD8+ (C) T lymphocytes were studied in multiple sclerosis patients lacking (M-, grey bars, n=191) and showing (M+, white bars, n=72) LS-OCMB, classified according to their age: ≤25, 26-30, 31-35, 36-40, 41-45, 46-50 and ≥51 years. ns, not significant. ****p < 0.0001.

FIGURE 3 | Changes in CSF CD4+ and CD8+ T lymphocytes producing cytokines in M- and M+ patients according to age. Absolute cell numbers (N.) of CD4+ (A–C) and CD8+ (D–F) T lymphocytes producing Interferon-gamma (IFNg), Tumor Necrosis Factor-alpha (TNFa) and Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF) in patients lacking (M-, n=105) and showing (M+, n=36) lipid-specific oligoclonal IgM bands, and classified according to their age in ≤45 years (white bars) and > 45 years (grey bars). ns, not significant; y, years. *p < 0.05; **p < 0.01.
However, none of these T cell subsets changed in M+ patients with age. Representative images of intracellular cytokine production by CD4+ and CD8+ T lymphocytes from M- patients are shown in Figure 4.

**Changes in Soluble Factors Associated With Immunosenescence and T Cell Exhaustion**

To gain insight into the regulation of adaptive immune cell activity, we analyzed CSF levels of PD-L1 and TIM-3, which are markers of T cell tolerance and exhaustion, respectively. We also explored serum titers of anti-CMV IgG, a marker of immunosenescence. PD-L1 and TIM-3 levels increased with age in M- and M+ patients (Table 2). The association with TIM-3 was lost in M- patients after adjusting for disease duration (Figures 1C, D and Supplementary Table 1). By contrast, serum anti-CMV IgG, a marker of immunosenescence, increased with age only in the M- group (Figures 1C, D and Supplementary Table 1).

**Soluble Factors Related to Innate Immune Response**

To further examine age-related differences between M+ and M- patients, we assessed CSF values of CHI3L1 and activin A, which are soluble factors related to the innate response. Activin A increased with age in both M- and M+ patients, while CHI3L1 levels only increased in the M- group. Notably, M+ patients had higher levels of this protein independently of age (Table 1). No significant correlations were lost after adjusting for disease duration (Figures 1C, D and Supplementary Table 1). By contrast, CSF NfL and C3 complement component levels did not change with age in M- or M+ patients (Table 2), but were higher in M+ patients independently of age (Table 1).

**Age and Disability Progression**

Finally, we explored the EDSS and MSSS scores. The first variable was studied in the total patient cohort, and the second one was studied in the 161 patients with at least six months of disease duration at lumbar puncture (120 M- and 41 M+). As reported above, M+ patients showed higher values of EDSS and MSSS scores (Table 1). When exploring the effect of age after adjusting for disease duration, we found increases in both scores in M- and M+ patients (Figures 1E, F and Supplementary Table 1).

**DISCUSSION**

Immunosenescence is characterized by a chronic activation of the innate immune response and reduced effectiveness of the adaptive response, which commonly occur after the age of 65 years (6). Converging evidence suggests that this process can occur earlier in patients with chronic immune-system activation, such as those with acquired immunodeficiency syndrome or rheumatoid arthritis (21, 22). In MS, older age affects the response to treatments and increases the risk of side effects (23, 24).
Pathological studies involving patient necropsies have provided recent reports on the molecular mechanisms involved in these changes came (25). These studies found an increase in innate immune-cell activation restricted to the CNS and a decrease in lymphocyte influx into the CNS in progressive forms of the disease (26). However, MS is a heterogeneous disease, and this process could not be uniform in all patients. Along these lines, it was shown that LS-OCMB (which is associated with a worse course of MS (16, 26) and higher inflammation demonstrated by augmented NfL values (27) have a protective effect on PML risk in patients receiving natalizumab treatment, independently of age (17, 28). This could imply that M+ status can modulate the effect of age in MS.

We aimed to explore this idea in 263 consecutive MS patients who had not previously received any disease-modifying treatment. M- patients showed a modest age-associated increase of PD-L1, a check point molecule capable of inducing T cell tolerization that rises in old individuals (29). This con

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study the innate immune system. LV designed and supervised the study. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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