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Mu-opioid receptor expression on B cells as a potential biomarker for chronic pain: a follow-up study with patients with fibromyalgia

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Abstract

Introduction: Before COVID-19 pandemic, we identified the percentage of B cells expressing the Mu-opioid receptor (Mu+ B cells) as a potential marker, named Mu-Lympho-Marker (MLM), for chronic pain (CP) in patients with fibromyalgia (FM) and osteoarthritis.

Objectives: Here, we demonstrate the stability of MLM over time through a comparative analysis of biological, clinical, and psychological data collected from a subgroup of patients with FM across 2 distinct research periods.

Methods: This is an observational, longitudinal study. Fibromyalgia participants enrolled in the first study were called back for follow-up sampling. Clinical data were recorded. Pain score was reported using the Numerical Rating Scale. Mu+ B cells percentage of expression was analyzed by flow cytometry. Immunofluorescence analyses were performed to explore the cellular localization of Mu-opioid receptor. Pain-free subjects served as control group. All the participants filled out self-reported psychological tests. Data were statistically analyzed.

Results: Mu+ B cells percentage of expression was constant in patients with FM, who consistently showed lower values than control group after 2 years (difference in the mean: 32.0 ± 4.4, $t = 7.330$, IC 95% [23.2–40.9], $P < 0.0001$). Confocal microscopy analyses revealed Mu cytoplasmic localization in patients with FM. We observed no significant changes between psychological outcomes during the 2 phases of the study, nor did we find any correlations with biological findings.

Conclusion: Mu-Lympho-Marker could be a promising marker for CP, as seen in FM cohort, and could be helpful for accurate diagnosis and tailored rehabilitation strategies. Further studies are needed to study MLM in CP of different aetiologies.

Keywords: Mu-opioid receptor, Pain markers, Chronic pain diagnosis, Chronic pain biomarkers, Fibromyalgia, B lymphocytes

1. Introduction

Chronic pain (CP) is a complex and multifaceted condition affecting millions of individuals worldwide,[45] with a serious impact on quality of life and health care systems.[52] Among the various conditions CP can refer to in clinical practice,[36] fibromyalgia (FM) serves as a prototypical example, characterized by persistent widespread pain and thus considered as a chronic primary pain syndrome.[32,47] Fibromyalgia affects between 0.2% and 6.6% of

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the global population, with a higher prevalence among women in middle age.[19,44,49] Beyond CP, FM is characterized by several debilitating comorbidities as well as, chronic fatigue, sleep disturbances, migraine, obesity, rheumatologic conditions, major depression, thyroiditis.[48] Given the substantial variability in clinical presentation,[43,48] it has been suggested that FM may be better conceptualized as a heterogeneous group of disorders rather than a single disease entity.[13] Moreover, FM appears to be influenced by a combination of genetic and environmental factors, including psychological and physical stressors, as well as various biological mediators such as neurotransmitters, hormones, cytokines, and elements of endocrine and immune pathways.[14,51]

Due to the uncertainty surrounding its diagnosis, the diverse range of symptoms, frequent presence of comorbidities, and challenges in treatment, FM is regarded as one of the most complex widespread pain conditions to manage.[10]

The absence of a dependable diagnostic test represents a significant obstacle in advancing the comprehension of CP[33] and in this specific contest of FM pain condition. Adding to the complexity, the lack of specific biomarkers for CP further hampers the FM treatment[40] and often causes tensions between patients and physicians. This can lead patients to believe their clinical condition is being misunderstood or dismissed as emotional issues, resulting in worsened disease severity and quality of life. Consequently, patients may engage in "doctor-shopping," which increases health care costs.[15] Consequently, the syndrome typically follows a persistent course without remission.[51]

Referring to CP as the primary and indispensable clinical characteristics of FM diagnosis, in the search for a reliable pain biomarker, Niculescu et al.[33] introduced a potential blood biomarker gene expression signature using microarray analysis. They identified pain "risk genes" that were more highly expressed in severe pain states than putative protective or resilience genes, concluding that a database of pain marker gene expression could hold significant importance for drug repurposing analysis and for the selection of new drug candidates. In addition, innovative molecular analyses, such as those involving microRNAs[23] and metabolic assessments utilizing vibrational spectroscopy techniques for constructing a pain "molecular fingerprint,"[18] are producing findings to advance pain biomarker research. It has also been proven that the integration of blood sample analysis with noninvasive functional brain imaging could be important in supporting clinical pain assessment and distinguishing between various CP conditions that may share similar features, such as FM, osteoarthritis (OA), and low back pain (LBP).[29,41]

Interestingly, in the pursuit of identifying specific markers derived from blood samples, the opioidergic system emerges as a particularly intriguing target owing to its interactions with 2 pivotal alarm systems associated with health risk factors, which are the nociceptive and the immune ones. Sensory and immune cells communicate by sharing same mediators and receptors.[25] In particular, opioid receptors are expressed by central and peripheral neurons, by neuroendocrine (pituitary, adrenals), immune, and ectodermal cells.[42] Due to the interaction between opioid peptides and specific receptors on leukocytes and sensory neurons, authors have likened the immune system to the sixth or seventh sense.[22]

Before the onset of the Covid-19 pandemic, Raffaeli et al. explored the Mu-opioid receptor's percentage of expression on immune cells as a potential CP diagnostic biomarker, referred to as Mu-Lympho-Marker (MLM). The authors observed that MLM on B cells, in painful patients with FM and OA, was statistically lower than in pain-free subjects. Importantly, this reduction in Mu-positive B-cell expression was not associated with the investigated psychological characteristics.[35] Raffaeli hypothesized the "Mu-opioid receptor theory," proposing that a reduction in the reserve of Mu-opioid receptors on immune cells could lead to a disruption of endogenous analgesic activity, thereby contributing to the onset and progression of pain.[24] These findings underscored the clinical imperative of identifying reliable pain biomarkers, with a focus on the interplay between the nervous system and peripheral opioid receptors pathway.[25]

For a biomarker to be considered effective, it should be easily and quickly detectable with low experimental costs and stabile over time under fixed physiopathological conditions.[27] Changes in marker expression, resulting from pharmacological treatment or pathological circumstances, should correspond to specific diagnostic or prognostic information that proves valuable in pain care therapy and rehabilitation.[26] Thus, to assess the stability of MLM on B cells, we analyzed it again in the previously prepandemic enrolled patients with FM, considering comorbidities and psychological variables. We compared the percentage of expression of MLM on B cells before (T0) and after a 2-year interval, during which widespread pain secondary to fibromyalgia persisted (T1). We aimed also to correlate clinical, biological, and psychological results obtained from self-reported standardized questionnaires in this time span, coinciding with the years in which Sars-Cov-2 epidemic was prevalent across Europe, including Italy.

2. Methods

2.1. Trial design

This is an observational, longitudinal single blind diagnostic trial.

The protocol has been designed by ISAL Foundation, the IRC FSH-Department of Health Science of University of Catanzaro, the Rheumatology Unit of Sapienza-University of Rome, and IRCCS San Raffaele Roma.

The current study has been approved by the institutional independent ethics committee of Sapienza University of Rome, with the name "I markers Bio-Psico-Sociali nella syndrome fibromialgica" (Fibromyalgia syndrome Bio-Psycho-Social markers), on March the 8, 2018 (Ref. 4937), and the trial has been registered in ISRCTN registry, ID: ISRCTN24645566, December 10, 2018.

2.2. Participants

The study involved patients with FM following both 1990 and 2010 American College of Rheumatology criteria[6,21] referred to the Clinic for the Diagnosis and Therapy of Fibromyalgia, Rheumatology Unit, Sapienza University of Rome (AOU Policlinico Umberto I, Rome, Italy). Patients with fibromyalgia assuming opioids, starting from 3 months before the enrolment and throughout the 2 phases of the study, were excluded from the trials, considering that opioid receptors expression and activity could be influenced by opioids medication. During the pandemic, medical examinations were less frequent; however, patients who self-certified that they had not taken opioids for at least 3 months were enrolled in the study. Patients with rheumatic pathologies were also excluded.

Pain-free healthy individuals, monitored through workplace health programs of AOU Policlinico Umberto I, were enrolled as control group (Ctrl(-)), matching the experimental group patients in age and gender. Ctrl(-) subjects were required to be free of any

acute painful conditions at the time of the study. Individuals with pathological conditions characterized by persistent and/or intermittent chronic pain and with a history of opioids consumption, at least from 3 months before the enrollment, were excluded (**Table 1**). Participants signed a specific informed consent form and General Data Protection Regulation obligations during the first clinical examination. Clinical data and pharmacological therapy were collected using a designated ad-hoc Case Report Form. Only the medical doctor could access patient names, to protect confidentiality.

2.3. Clinical and psychological assessments

All the patients were called-back for the follow-up study, from February till December 2021. Pain score was reported using thea 11-points Numerical Rating Scale (NRS), with 0 for "no pain" and 10 for "the worst possible pain."[7] As during the first prepandemic blood sampling (T0), the Italian version of the following self-report tests was administered to all the patients: (1) The Fibromyalgia Impact Questionnaire (FIQ)[38] for functional disability, consisting of 10-item measuring physical functioning, work status, depression, anxiety, sleep, pain, stiffness, fatigue, and well-being; (2) The Illness Perception Questionnaire-Revised,[16] which consists of 38-items evaluating emotional impact, control, understanding, and perceived duration of the illness; (3) The Coping Strategies Questionnaire,[28] consisting of 27-items measuring 6 different coping strategies as self-affirmation, distancing, praying, denial, distraction, and catastrophizing; (4) The Depression, Anxiety and Stress Scale-21,[5] a 21-items measure of psychological distress in depressive, anxious, and stress-related symptoms; and (5) The CP Acceptance Questionnaire,[3] which consists of 20-items measuring pain acceptance conceptualized as the attitude of persisting in doing pleasant activities instead of trying to control pain. At T1, we also administered the Revised version of FIQ Questionnaire (FIQ-R), a specific tool proposed to overcome the limits of the original FIQ[2] and improve the questionnaire with new questions, concerning mental sensitivity, memory, and environmental equilibrium.[37]

2.4. Blood collection

Fifteen milliliters of peripheral blood were collected during clinical examination, following the standard protocol,[12] to be analyzed within the next 24 hours. Research biologists were blind to patients' personal information and therapy.

2.5. Flow cytometry analysis

Two hundred microliters of peripheral blood samples were stained, for 20 minutes, with the following antibodies: APC-conjugated anti-Mu (LSBio), BUV395-conjugated anti-CD45 (Becton Dickinson [BD]), BV480-conjugated anti-CD3 (BD), and BV785-conjugated anti CD-19 (BD), for 20 minutes at 4°C. After incubation at room temperature, for 15 minutes, with BD FACS Lysing Solution (BD) stained samples were centrifuged for 5 minutes at 1500 RPM. After washing, samples were suspended in PBS and then acquired on LSR Fortessa X20 flow cytometer (BD). Analyses were performed using Diva software, to detect the percentage of Mu+ B cells in FM and pain-free control groups.

2.6. Lymphocytes isolation

Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll (GE Healthcare, Milan) density centrifugation gradient. After dilution with PBS, 15 mL of whole blood sample were layered over 15 mL of Ficoll medium and centrifuged at 2200 RPM for 20 minutes (maximum acceleration, without brake). The resulting PBMCs were washed with PBS and centrifuged at 1800 RPM for 5 minutes, acceleration and deceleration max. Pellet was suspended with PBS and centrifuged at 800 PRM for 10 minutes. Obtained PBMCs were used for immunofluorescence analysis.

2.7. Immunofluorescence analysis

Coverslips were cross linked with polylysine 10 mg/mL for 50 minutes under UV, and then PBMCs were seeding on coverslips (24 hours). Cells were fixed for 10 minutes at 37°C in 4% paraformaldehyde in PBS, containing 4% sucrose, permeabilized with 0.1% Triton X-100 in PBS, for 4 minutes, at room temperature and washed 3 times for 5 minutes with PBS. All antibodies were diluted in PBS containing 3% bovine serum albumin. The following primary antibodies were used (60 minutes, 37°C): APC-conjugated anti-Mu (LSBio) and BV785-conjugated anti CD-19 (BD) (1:200). After the primary antibody incubation and subsequent rinsing, the coverslips were incubated with fluorescently labeled secondary antibodies, goat anti-rabbit IgG conjugated to Alexa Fluor 488 or goat anti-mouse IgG conjugated to Alexa Fluor 555 (Molecular Probes, Millipore, Burlington, MA), at a 1:500 dilution in 3% BSA. The specific combinations of secondary antibodies used were determined by the primary antibodies applied. Nuclei were counterstained with DAPI 1:1000 in PBS for 5 minutes RT (Sigma D9542). Coverslips were mounted in FluoromountTM Aqueous Mounting Medium (Sigma F4680), and samples were observed and acquired with confocal Nikon Eclipse Ti2 microscope, equipped with a VideoConfocal (ViCo) system. Co-localization between Mu and CD19 protein was performed using z-stack and Deconvolution Richardson-Lucy data from neighboring focal planes, to detect similarities between image intensities of 2 and potentially more fluorescently labeled biological entities.

Table 1

Inclusion and exclusion criteria.

	Inclusion criteria	Exclusion criteria
FM patients	Age: 18–65	Rheumatic pathologies
	1990 ACR criteria	Opioids consumption
	2010 ACR criteria	
Ctrl(-) pain-free subjects	Age: 18–65	AP at the enrollment
	Gender: Female	Persistent CP (last 3 mos)
	Source: Workplace health programs	Opioids consumption

Schematic description of inclusion and exclusion criteria for patients with FM and Ctrl(-) subjects.
ACR, American College of Rheumatology; AP, acute pain; CP, chronic pain; FM, fibromyalgia.

2.8. Statistical analysis

Statistical analyses were conducted using SPSS version 28 and Prism-GraphPad 8.0.2 software. Descriptive statistics were employed to summarize and describe continuous variables. Mean and standard error of the mean (SEM) were calculated for normally distributed biological variables. For categorical variables, frequency (count) and percentage were used to describe the distribution of different categories. In bivariate analysis, the χ^2 test was used to compare categorical data. Unpaired t tests and paired t tests were used to compare 2 groups (experimental group vs control group) and within-group variations (T0, T1), respectively, if normally distributed; otherwise, the Mann–Whitney and Wilcoxon Rank Test were applied. Pearson correlation coefficient was used to assess the existence of an association between quantitative variables. A two-sided *P*-value threshold of 0.05 was considered statistically significant.

3. Results

3.1. Participants

Of the 59 patients with FM enrolled in the initial study before the onset of the COVID-19 pandemic, 21 have participated at the follow-up study. The control group consisted of 43 pain-free subjects. The totality of the participants was female, ranked between 18 and 65 years old, and mean age 54 ± 8.8.

3.2. Pain intensity score

Patients' pain intensity score was detected at T1 using NRS scale, as already done at T0. All the patients ranked between moderate (NRS [4–6], n = 6.28, 5%) to severe (NRS [7–10], n = 15, 71.5%) pain, median (interquartile range [IQR]) 8.0 (7.0–9.0). The elapse of the time did not influence pain intensity score, considering that, at T0, the median (IQR) was 8.0 (8.0–9.0)[35] (Wilcoxon ranked test, Z = 1.635, *P* = 0.102) (**Fig. 1**). Control group subjects declared to be without any chronic pain (NRS = 0), at least from 3 months before enrolment. No participants contracted SARS-Cov-2 either before or during the study.

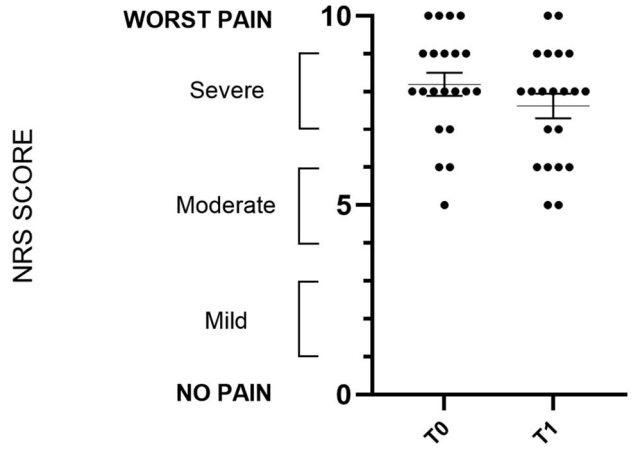

Figure 1. Pain intensity score (NRS). At the follow-up sampling (T1), all the patients experienced pain intensity ranking from moderate to severe, which was not statistically different from their pain intensity level before the pandemic (T0). T1 median (IQR): 8.0 (7.0–9.0), T0 median (IQR): 8.0 (8.0–9.0), Wilcoxon ranked test (Z = 1.635, *P* = 0.102). IQR, interquartile range; NRS, numerical rating scale.

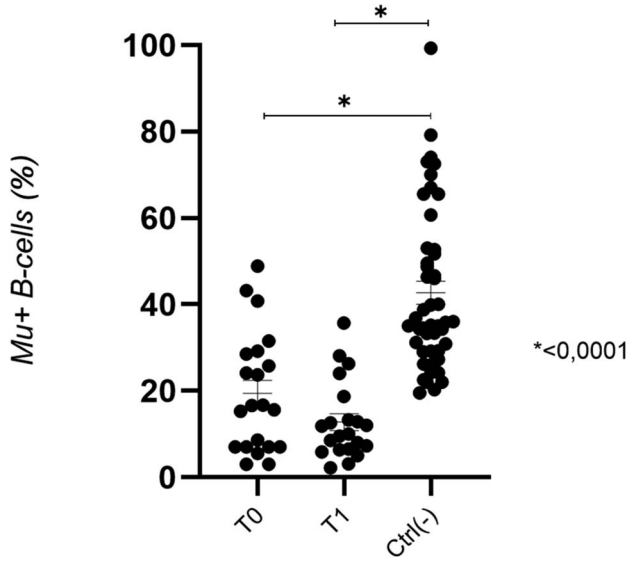

Figure 2. MLM on B cells. Comparison of MLM on B cells at T0 and T1: no significant differences were detected between T0 and T1 (Pair *t* test). MLM on B cells remains significantly lower in patients with FM compared to pain-free individuals at T1: T1 FM mean: 12.76 ± 1.9 vs Ctrl(-) mean: 42.71 ± 2.7 (32.0 ± 4.4, t = 7.330, IC 95% [23.2–40.9], *P* < 0.0001). Results are expressed as mean ± SEM. FM, fibromyalgia; MLM, Mu-Lympho-Marker.

3.3. Mu-lympho-marker on B cells

Blood samples were analyzed in flow cytometry to detect MLM on B cells in patients with FM and in pain-free subjects. We found intragroup homogeneity between FM and Ctrl(-) at T1 (FM: 12.76 ± 1.9 vs Ctrl(-) 42.71 ± 2.7, mean ± SEM; difference in the mean between the 2 groups: 32.0 ± 4.4, t = 7.330, IC 95% (23.2–40.9), *P* < 0.0001) as already found at T0.[35] We compared the mean of MLM on B cells at T0 (18.4 ± 2.5) with those measured during the pandemic period at T1 (12.76 ± 1.9), and we did not detect any significant difference (paired *t* test). Mu-lympho-marker expression remains stable over time (**Fig. 2**).

3.4. Mu-opioid receptor cellular localization

We performed confocal microscopy analysis, with PBMC from 4 patients with FM and 4 Ctrl(-) individuals, to localize Mu-opioid receptor in B cells and figure out whether it could be internalized in the cytoplasm, to explain its quantitative reduction in patients with FM in respect to Ctrl(-) subjects. Interestingly, **Figure 3** shows a cytoplasmic localization of Mu-opioid receptor, corresponding to its internalization in patients with FM, differently from Ctrl(-) subjects (**Fig. 3**).

3.5. Analysis of psychological results and mu-lympho-marker on B cells

We compared psychological questionnaires results, at T0 and T1, of all patients with FM analyzed at T1 and we did not find any significant change (paired *t* test, Supplementary Table 1, http://links.lww.com/PR9/A312). Thus, no significant variation in the scales score has been detected over time. We also correlated psychological questionnaires results, both at T0 (Supplementary Table 2, http://links.lww.com/PR9/A312) and T1 (Supplementary Table 3, http://links.lww.com/PR9/A312), with MLM on B cells at T1, and we did not find any significant correlation (Pearson correlation).

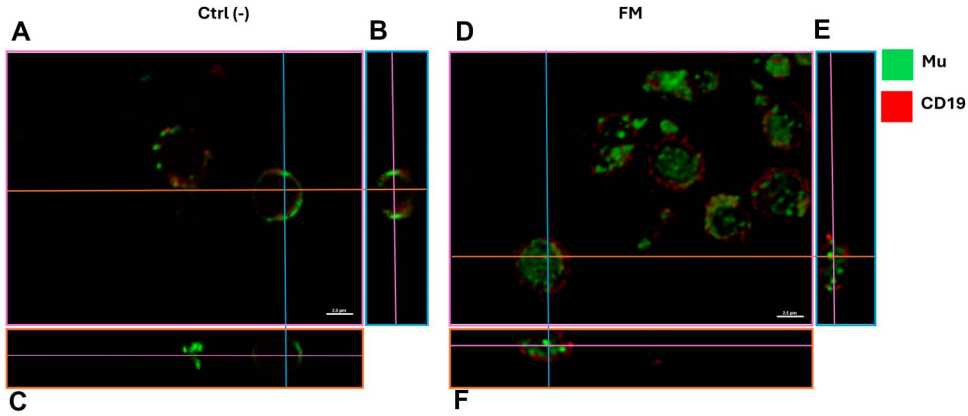

Figure 3. Mu-opioid receptor cellular localization. Confocal microscopy image of PBMCs from one Ctrl(-) subject (left) and one patient with FM (right) at ×60 magnification. (A–D) Side view of the z-stack, x and y plane; (B–E) side view of the z-stack, x and z plane; (C–F) side view of the z-stack, y and z plane. Z-stack images show B cells (CD19) and Mu receptor colocalization at the level of cytoplasmic membrane in Ctrl(-) sample, differently from FM sample where Mu is detected in a cytoplasmic localization. FM, fibromyalgia; PBMC, peripheral blood mononuclear cell.

4. Discussion

Fibromyalgia, always characterized by widespread CP throughout the body, has been included in the International Classification of Diseases (ICD-11) within the category of "chronic primary pain."[32] This concept encompasses pain persisting or recurring beyond 3 months without any specific cause. For CP, objective biomarkers are not currently available, elevating the risk of misdiagnosis.[2] Interestingly, it has been demonstrated that a correct diagnosis of CP, as for FM, could lead to economic savings, with a reduction in expenditure on tests, diagnostic imaging, pharmaceuticals, and visits to primary care physicians.[1]

Chronic pain biomarkers could play a crucial role in uncovering mechanisms, in identifying homogeneous subgroups also of patients with FM within heterogeneous pain conditions and offering insights into biological changes associated with the treatment.

We found that, in a CP state due to FM and OA, the percentage of B cells expressing Mu on their membranes was significantly lower than the same percentage in pain-free subjects.[35] This low expression could represent a possible biomarker of CP state and we named it "MLM."[24] In the present work, we studied MLM stability over time, especially during Covid-19 pandemic period, a very peculiar social and environmental historical moment that influenced life habits and quality worldwide. The CP population, who are usually more vulnerable than others, underwent varying degrees of conditioning with respect to social and psychological determinants of health.[11] In particular, patients with FM declared physical and mental worsening because of isolation and impossibility to attend medical examinations and subsequent pharmacological treatments and rehabilitation programs.[39]

In our study, blood sampling has been conducted during alternated quarantine periods, characterized by social limitation months. A total of 21 of 59 patients with FM enrolled at T0 decided to participate at the follow-up, attending the second blood sampling and self-filling psychological questionnaires. Everyone reported that they had not contracted a Sars-Cov-2 infection during that time frame. The remaining patients, almost the half of all the participants at T0, did not attend the routine clinical visits during the pandemic period, because of the fear to reach the hospital, considered as a possible hotspot of infection.[9]

We analyzed MLM on B cells to detect possible differences in FM patients' immune-phenotype, between T0 and T1. All patients with FM analyzed reported, at T1, a pain intensity score (NRS scale) that was not significantly different from the score reported in the first study at T0.

Interestingly, we did not detect any significant difference between Mu+ B cells percentage of expression tested before Covid-19 vs that one tested during the pandemic. The values in patients with FM at both T0 and T1 were statistically lower than those observed in pain-free individuals.

Mu-lympho-marker maintains stability over time, emerging as a reliable indicator of widespread CP in patients with FM. As already postulated in our first study,[35] moderate to severe pain intensity corresponds to a reduction in the percentage of Mu+ B cells. This deficit of immune cells presenting Mu receptor on the cellular membrane could affect pain perception, leading to an uncontrolled widespread pain condition.

In the present study, we performed immunofluorescence analysis to detect the cellular localization of Mu-opioid receptor in patients with FM and pain-free subjects. Confocal microscope images showed Mu cytoplasmic localization in B cells of patients with FM and Mu transmembrane localization in pain-free subjects. The receptor internalization is typically associated with tolerance, due to a prolonged exposure to opioid drugs.[30] Interestingly, we exclusively enrolled patients who had not undergone any opioid drug treatment throughout this timeframe, considering also that Mu mRNA expression level in lymphocytes could be altered by exogenous opioids administration, such as morphine or even more morphine plus bupivacaine.[8]

However, it has instead been demonstrated that a continuous availability of endogenous opioids enhances recycling and maintains the signaling of Mu receptor, counteracting the onset of peripheral opioid tolerance.[20,53] Thus, we could hypothesize Mu internalization due to a possible low concentration of β-endorphin opioids peptides in patients with FM, as suggested by Bidari et al.[4]

In accordance to this hypothesis, in patients experiencing chronic neuropathic pain after trauma or surgery, it has been found a lower level of beta-endorphin than in healthy subjects, too.[31] These findings were also observed in chronic fatigue syndrome and patients with FM, who presented lower concentrations of PBMC β-endorphin when compared to pain-free individuals.[34] Moreover, a preclinical study using a knockout mice approach has showed a reduction of Mu-opioid receptors in mice lacking either proencephalin, β-endorphin, or both.[17] Authors suggested a regulation of opioid receptors expression leading by

opioid peptides, involving transcriptional factors.[50] In the specific, opioid peptides could induce the expression of chaperons, or either can act themselves as chaperones, thus controlling the receptors folding and packaging.[46]

Further studies concerning endogenous opioid peptides, in patients affecting by FM and other CP conditions, could help to better understand and confirm this hypothesis.

In the current study, we have analyzed patients' psychological characteristics, before and during the pandemic, also in relation to biological findings. Fibromyalgia symptoms severity could be amplified by psychological stress, suggesting to physicians a better patients monitoring also of mental stressors.[9] The lack of significant changes between psychological questionnaire results, from T0 to T1, may suggest a stability in patients' psychological conditions, despite this timeframe. Such results could stem from the fact that all the patients participating in the follow-up were experiencing moderate to severe pain at both T0 and T1, without any significant change in NRS score. It is, therefore, reasonable to consider that the stability of MLM and pain intensity over time corresponds to stability in patients' psychosocial conditions, and that changes in the former variables could correspond to variations in the latter.

5. Conclusion

The stability of MLM over an extended period, characterized by significant bioimmune and psychological stress, such as the Covid-19 pandemic period, leads us to hypothesize its suitability as a putative biomarker of CP in patients with FM. Extending this discussion beyond FM, objective pain assessment through MLM analysis could be of significant value in the assessment of CP in cases where traditional self-reporting is challenging. This includes people with cognitive/communicative impairments or psychiatric disorders for whom it is particularly difficult to obtain a reliable and objective assessment of pain.

Further studies are necessary to understand the biological and genetic bases underlying Mu-opioid receptor underexpression on immune cells and to define its sensitivity and predictive reliability as a diagnostic marker, in different CP syndromes. Thus, we aim to achieve an even more precise understanding of MLM's role in clinical practice, pain management, and rehabilitation strategies.

Disclosures

The authors declare no conflict of interest. The funders had no role in the design of the study, in the collection, analyses, or interpretation of data, in the writing of the manuscript, or in the decision to publish the results.

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