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Peripheral immune aberrations in fibromyalgia: A systematic review, meta-analysis and meta-regression

Laura Andrés-Rodríguez[1,2,3,4†], Xavier Borràs[1,4†], Albert Feliu-Soler[1,2,3,4*], Adrián Pérez-Aranda[1,2,3*], Natalia Angarita-Osorio[1,2,4], Patrícia Moreno-Peral[3,5], Jesús Montero-Marin[3], Javier García-Campayo[3,6], Andre F. Carvalho[7,8], Michael Maes[9*], Juan V. Luciano[1,2,3*]

[1] Group of Psychological Research in Fibromyalgia & Chronic Pain (AGORA). Institut de Recerca Sant Joan de Déu. C/ Santa Rosa 39-57, 08950. Esplugues de Llobregat, Spain.

[2] Teaching, Research & Innovation Unit, Parc Sanitari Sant Joan de Déu. C/ Dr. Antoni Pujadas 42, 08830. Sant Boi de Llobregat, Spain.

[3] Primary Care Prevention and Health Promotion Research Network, RedIAPP. Madrid, Spain.

[4] Faculty of Psychology, Universitat Autònoma de Barcelona. Bellaterra (Cerdanyola del Vallès), Barcelona, Spain.

[5]Instituto de Investigación Biomédica de Málaga (IBIMA), Distrito Sanitario Málaga-Guadalhorce. Málaga, Spain.

[6]Department of Psychiatry, Miguel Servet Hospital, Aragon Institute of Health Sciences (I+CS). Zaragoza, Spain.

[7]Department of Psychiatry, University of Toronto, Toronto, ON, Canada.

[8]Centre for Addiction and Mental Health (CAMH), Toronto, ON, Canada.

[9]Department of Psychiatry, Chulalongkorn University. Bangkok, Thailand.

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† Both authors contributed equally to this paper and should be considered as co-first authors.

* Corresponding author.

Teaching, Research & Innovation Unit, Parc Sanitari Sant Joan de Déu.

C/ Dr. Antoni Pujadas 42, 08830, St. Boi de Llobregat, Spain.

Tel: +34 936406350 (Ext. 1-2540).

E-mail: a.feliu@pssjd.org (Albert Feliu-Soler), ap.aranda@pssjf.org (Adrián Pérez-Aranda), dr.michaelmaes@hotmail.com (Michael Maes) jvluciano@pssjd.org (Juan V Luciano)

Abstract

The objective was to identify immune alterations in patients with fibromyalgia syndrome (FMS) compared to healthy controls (HC) using meta-analysis and meta-regression. Six electronic databases were searched for suitable original articles investigating immune biomarkers in FMS in comparison to HC. We extracted outcomes and variables of interest, such as mean and SD of peripheral blood immune biomarkers, age or sex. A random-effects model with restricted maximum-likelihood estimator was used to compute effect sizes (standardized mean difference and 95% CI, Hedges' *g*) and meta-analysis, group meta-analysis and meta-regressions were conducted. Forty-three papers were included in this systematic review, of which 29 were suitable for meta-analysis. Interleukin (IL)-6 (g=0.36 (0.09-0.63); I^2=85.94; p=0.01), IL-4 (g=0.50 (0.03-0.98); I^2=81.87; p=0.04), and IL-17A (g=0.53 (0.00-1.06); I^2=87.15; p=0.05), were significantly higher in FMS compared to HC while also combinations of cytokines into relevant phenotypes were significantly upregulated including M1 macrophage (g=0.23 (0.03-0.43); I^2=77.62; p=0.02), and immune-regulatory (g=0.40 (0.09-0.72); I^2=84.81; p=0.01) phenotypes. Heterogeneity levels were very high and subgroup and meta-regression analyses showed that many covariates explained part of the heterogeneity including medication washout, sex, time of blood sampling and exclusion of patients with major depressive disorder. In conclusion, FMS is accompanied by a disbalance between upregulated pro-inflammatory (M1 and Th-17) and immune-regulatory cytokines although effect sizes are small-to-moderate. Based on our results we provide specific methodological suggestions for future research, which should assess Th-1, Th-17, chemokines, and Th-2 phenotypes while controlling for possible confounding variables specified in this study.

Key words: Fibromyalgia, Cytokines, Chemokines, Immune biomarkers, Meta-analysis

1. Introduction

Fibromyalgia syndrome (FMS) is a disabling condition mainly characterized by chronic widespread musculoskeletal pain. Other common symptoms are stiffness, fatigue, sleep problems, mood disturbances, distress, and perceived cognitive impairment, commonly known as "fibrofog" (Feliu-Soler et al., 2018; Häuser et al., 2015; Wolfe et al., 2016). Although the pathophysiology of FMS is unclear, it is commonly classified as a Central Sensitization Syndrome, defined as nociception-driven amplification of neural signalling within the central nervous system leading to pain hypersensitivity (Bäckryd et al., 2017; Woolf, 2011).

There is an increasing interest in characterizing the biological hallmarks of FMS, mostly focusing on immune biomarkers of cell-mediated immunity (CMI), which includes the interactions between inflammatory M1 macrophage (involving interleukin (IL)-6 and IL-1β) and T helper (Th)-1 (including IL-2 or interferon (IFN)-γ) cytokines. Other studies also measured CMI pathways coupled with increased levels of acute-phase reactants including high-sensitivity C-reactive protein (hs-CRP), and chemokines including CXCL-8, CXCL-11 or CX3CL1), which all together may be conceptualized as the immune-inflammatory response system (IRS). On the other hand, the compensatory immune-regulatory system (CIRS) has been of special interest since T-regulatory (T-reg) and Th-2 cells produce anti-inflammatory cytokines including IL-10 and IL-4, which may attenuate the IRS through multiple feedback signals (Maes and Carvalho, 2018). Importantly, M1 and Th-1 related cytokines play a critical role in the generation of both acute and chronic pain (Feinberg et al., 2017; Sluka and Clauw, 2016). As such, one of the hypothesis posits that low-grade chronic inflammation may play an important role in the pathophysiology and maintenance of FMS at least in a subgroup of patients (Gür et al., 2002). Nevertheless, FMS research focused on a limited number of IRS/CIRS biomarkers including IL-1β, IL-6, IL-10, CXCL-8 and tumor

necrosis factor (TNF)-α and did not include other major players including soluble IL-6 receptor (sIL-6R) and sgp130 levels to assess IL-6 trans-signalling, excitotoxic tryptophan catabolites, IL-5 and CCL-11. Further explanation of the periperal immune phenotypes can be found in the electronic supplementary files (ESF1 Table 1).

Furthermore, FMS as currently defined is rather unspecific as many pain and fatigue-like symptoms with unknown origin (Häuser et al., 2017) may be classified as FMS, causing heterogenicity when characterizing the possible immune features of FMS. In addition, a major flaw of immune research in FMS is the lack of control for analytical and biological variability in immune markers and the high heterogeneity in methodological designs. Immune biomarkers are affected by a large number of confounding variables such as comorbid pathologies or infections, treatments modulating immune functions including use of antidepressants (ADs) (Hannestad et al., 2011), life-style behaviours including diet, physical exercise, smoking and intake of antioxidants including vitamin D (Kiecolt-Glaser and Glaser, 1988; von Känel et al., 2014), age and sex (Kiecolt-Glaser and Glaser, 1988), body mass index (BMI) (Feinberg et al., 2017), phase of the menstrual cycle (O'Brien et al., 2007), emotional status (Köhler et al., 2017) and seasonal and diurnal variation (Nilsonne et al., 2016). Most published studies in FMS did not use a standardized methodology to control for the above-mentioned variables when analysing the results. Published systematic reviews and meta-analysis on this topic concluded that immune research in FMS needs substantial improvement by controlling for those confounders (Rodriguez-Pinto et al., 2014; Üçeyler et al., 2011). A recent narrative review underscored the large variability in results but also concluded that cytokines and chemokines emerge as possible biomarkers for FMS (Rodriguez-Pinto et al., 2014).

Hence, the main objectives of this study were a) to systematically review and meta-analyse evidence on IRS/CIRS biomarkers distribution in FMS patients in comparison with

healthy controls (HC); and b) to assess the degree of between-studies heterogeneity and delineate possible confounders that may impact heterogeneity.

2. Materials and Methods

The review protocol was registered at PROSPERO in December 18, 2017 (registration number: CRD42017080290). The review protocol was registered at PROSPERO in December 18, 2017 (registration number: CRD42017080290). Few changes have been made since the publication of the protocol, as for example the incorporation of two new expert co-authors and the resignation of one of the original authors. We also modified the inclusion criteria, since we were able to include papers written in French and German. On the other hand, we did not use the NOS scale to evaluate risk of bias, and instead created our own scale (see below). Finally, we were able to conduct meta-regression analyses, which were not planned in the protocol. This systematic review and meta-analysis complied with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement (Liberati et al., 2009).

2.1 *Literature search*.

Searches were conducted in PubMed, EMBASE, PsycINFO, Scopus, Cochrane and Web of Science using free text and thesaurus alone and in combination. The main search terms were "fibromyalgia", "inflamm*", "immun*" and the specific names (and acronyms) of the IRS/CIRS markers. The following search string for PubMed was used and adapted to other database formats: ((inflamm* OR immun*) AND (cytokine OR chemokine OR IL-6 OR IL-1β OR interleukin OR (C-Reactive Protein OR CRP) OR (tumor necrosis factor OR TNF) OR (interferon OR IFN) OR (transforming growth factor OR TGF) OR lymphocyte OR Macrophage OR Microglia OR neutrophil OR east cells OR Eosinophil OR Basophil OR monocyte OR leukocyte* OR dendritic) AND (fibromyalgia)), with filters activated:

Publication date from 1990/01/01 to 2017/12/31 (search updated in March 25th 2019), Humans (see **ESF1 Table 2**).

We restricted the search to papers written in English, Spanish, French or German and examined the reference lists and conducted a reverse citation search of included papers. Clinical experts in both FMS and immune markers of the review team were also asked to help uncover any additional literature that was not identified from the previously specified searches.

Inclusion criteria were: cross-sectional studies and baseline data from longitudinal studies, with a HC group, in English, Spanish, French or German; diagnosis of FMS using ACR 1990 or 2010 criteria, adults (≥ 18 years); peripheral immune biomarkers (both in serum or plasma). Studies that examined the effects of interventions on immune biomarker levels were included only when the baseline data were available, or the authors provided the data. We excluded studies, which examined samples other than serum and plasma including stimulated whole blood or PBMCs. We also excluded studies with comorbid conditions other than Major Depressive Disorder (MDD) and those that did not include a HC group. We only included studies in which the healthy control group was well-defined by the absence of any significant disease including chronic pain. Reviews, systematic reviews, and meta-analysis papers, along with grey literature were only used to check the reference list in order to be sure that no study was left out.

2.2 Study selection.

The studies were initially screened on the basis of their titles and abstracts by two authors (LA-R and AP-A) and when there was a disagreement, a third author (AF-S) helped to reach a consensus. Duplications were removed.

In a subsequent phase, the full-text of the resulting list of papers were examined to check study eligibility. Again, extraction of data from full-length papers was independently

carried out by two researchers (LA-R and AP-A), being key information from each study entered into a predefined form.

2.3 Data abstraction and Study Coding.

The first author (LA-R) summarized all information on the studies in a Comprehensive Meta-Analysis (CMA V3) spreadsheet while the fourth author (AP-A) checked the extracted data. The following information was collected from each included study: sample size in the study groups, mean and SD of biomarkers´ levels, % of women, and mean age. We registered year of publication, diagnostic criteria, duration of illness (in years), and severity of FMS (Fibromyalgia Impact Questionnaire, or Visual Analogue Scale). With regard to the immune biomarkers, we registered the blood extraction time, whether plasma or serum was employed as well as the type of assay used to quantify biomarker levels. Additionally, we registered other important variables when available including mean BMI, exclusion of MDD, medication washout, and latitude of the country where the study was conducted.

2.4 Methodological quality of the included studies and Immune Confounders Scale (ICS).

We developed a checklist (see **ESF1 Table 3**) to evaluate the methodological quality of studies reporting IRS/CIRS in FMS. In order to obtain a quality score, two authors (LA-R and XB) independently completed the checklist, while a third reviewer was consulted (AF-S) in case of disagreement. The checklist comprises two parts: the first part aims to assess methodological quality of immune-related studies, considering the more critical aspects including sample size, matched groups, reporting detection limit, etc. A score is calculated to estimate the methodological quality which may vary from 0 to 10, with higher scores indicating better methodological quality. The second part includes a red-points scale penalizing the disregard of critical confounders, which are known to induce a considerable

heterogeneity in immune-related studies. Here total scores may range from 0 to 29, with 0 indicating that all the confounder variables were taken into account (e.g matching the study groups, met all exclusion criteria or statistically adjusted for background variables) and 29 when there was no control at all.

2.5 Data analyses

We used the CMA V3 software. A meta-analysis was executed whenever values of immune markers were available in three or more studies. When data of immune markers were available in less than three studies, they were still included in the immune phenotype analysis. Since we expected differences of measurement methods between the studies, we estimated standardized mean difference (SMD) and 95% CI (Hedges's g) for each immune mediator, which provides an unbiased effect size (ES) adjusted for small sample sizes. Studies were considered outliers and removed when SD > 4.0 (Sephton and Spiegel, 2003). The significance was set at $p < 0.05$ (two sided tests), the cut-off points for valuing the ES were 0.2 (small), 0.5 (moderate) and 0.8 (large) (Cohen, 1988). We selected the random-effects model with restricted maximum-likelihood for the study under the assumption that population's characteristics in the different studies may differ from each other. Egger's linear regression test was used to detect publication bias. This test probes for asymmetry of the funnel plot at $p < 0.10$ showing significant asymmetry and therefore publication bias. When Egger's linear regression test presented a significant asymmetry, the Duval and Tweedie's trim-and-fill procedure was used to estimate the ES adjusting for publication bias. Heterogeneity between studies was assessed using the Cochran Q test, and I^2 statistic to indicate the percentage of total variation across several studies due to heterogeneity and it is considered high when ≥ 50% (Patsopoulos et al., 2009). Potential sources of heterogeneity across studies for each ES estimate were explored when at least 10 studies reported data of the same variable, using either subgroup meta-analysis (with a minimum of 3 studies per sub-

group) or random-effects meta-regression analyses (Köhler et al., 2017) while the group-by analyses were checked using within- and between-group heterogeneity results. Moreover, all meta-analysis were examined using the leave-one-out sensitivity analysis.

The following variables were considered in the group meta-analysis: exclusion of comorbid MDD (yes/no), medication washout (yes/no), time of blood sample (morning vs. not reported), sex (100% woman vs. mixed samples), total scores on the two sub-scales of the ICS dichotomized using the median-split method, age differences (FMS minus HC, dichotomized using less vs. more than one year), ethnicity, type of assay (ELISA vs. Others), use of serum or plasma, latitude dichotomized using 40º, and year of publication dichotomized using 2010 as threshold (the year that the ACR revised the diagnostic criteria for FMS). In the meta-regression, we used % of females in the whole sample, year of publication and total scores of the two sub-scales of the ICS as covariates (continuous regressors). The confounder variables used in our study were selected by our immune experts and comprised those variables that are known to affect the immune status and those that were reported in a sufficient number of papers to perform group meta-analysis or meta-regression.

Unfortunately, there were not enough studies reporting BMI, severity of illness, years with FMS, diet, physical exercise, smoking, use of vitamin D or antioxidant supplements, menstrual cycle, anxiety or depression to evaluate the effect of these variables.

Moreover, immune biomarkers were grouped (Maes and Carvalho, 2018) in order to evaluate the following phenotypes: M1 (IL-6, s-IL-6R, TNF-α, IL-1β, IL-1RA, MCP-1 and MIP-1), Th-1 (IL-2, IL-12, sCD8, IFN-γ), CMI (M1+Th-1), IRS (CMI, Chemokines, IL-7, IL-15, IL-17A, IL-18, IL-31, IL-33, HGMB1 and hs-CRP) and CIRS (IL-4, IL-5, IL-13, sTNF-α R1, sTNF-α R2, IL-1RA, sgp130 and IL-10).

2.6 Patient and public involvement

No public or patient representatives were directly involved in the draft or process of this review.

3. Results

3.1 Selection and inclusion of studies

The initial literature search on the electronic databases yielded 1,520 potentially relevant studies whereas 6 additional papers were identified through other sources (751 abstracts in total, after removal of duplicates). Seventeen out of the 60 full-text articles were excluded because they did not meet the inclusion criteria. Therefore, 43 studies complied with the inclusion criteria and were included in the systematic review involving 2 randomized control trials, 1 non-randomized control trial, 1 cohort study and 37 case-control studies. No unpublished relevant studies were found. Of these 43 studies that matched our inclusion criteria we were able to obtain data from 29 studies to conduct the meta-analysis. One study was excluded given that the meta-analysis results for TNF-α (the only assessed immune biomarker in that study) were considered to be an outlier (> 4SD) (Cordero et al., 2013) (see **Figure 1**). Nine articles could not be included because data were not provided upon request, and 4 because they did not have files of the requested data anymore (see **ESF1 Table 3**).

Insert Figure 1 around here

3.2 Characteristics of the included studies in the systematic review

The sample sizes of the study groups ranged from 16 to 250 participants. The included studies were conducted in 12 different countries: Spain (n=10), USA (n=8), Turkey (n=6), Germany (n=5), Brazil (n=4), Sweden (n=3), Italy (n=2), Belgium (n=1), Mexico (n=1), India (n=1), Korea (n=1) and Israel (n=1). We found that 74.4% of the studies included in our systematic review had matched participants by age and gender, and that 76.7% of them controlled the results for potential confounders (e.g. age, sex, BMI). We found that 58.1% of the studies specified the time of sample collection, 30.2% specified that

participants were free of ADs while 74.4% reported that participants were free of medication. 41.9% of the papers reported durations of illness and up to 97.7% of the studies reported the name of the manufacturer of the test kits used to assay the biomarkers (but only around 35% reported how they handled data under the detection limit). All studies reported the blood fraction on which the assays were conducted. Further information of the systematically reviewed characteristics of individual studies is detailed in **ESF1 Table 4**. Applying the ICS we found that 16.3% of the studies included in this systematic review presented low methodological quality (0-4), 62.8% of them have moderate methodological quality (5-7), and only 20.9% had a high methodological quality (8-10). Additionally, 79.1% of the papers did not asses or control for at least 10 of the 20 well established confounder variables. These results are available upon request.

3.3 Meta-analysis

Analyses were performed with and without outliers, which were detected in two instances. In the case of IL-10, we removed Malhorta et al. (2012) because the g value was -13.56 indicating a clear bias in the HC direction. In the case of TNF-α, we removed Cordero et al. (2013) because the g value was 8.59, which was in the FMS direction.

3.3.1 Individual Immune biomarkers

IL-6, IL-4 and IL-17A (**Table 1, Figure 2**) levels were higher in FMS patients than in HC, with small to moderate effect sizes (*g*s ranging from 0.36 to 0.53). Marginally significant differences ($p < 0.10$) were found for sIL-1RA, CXCL-11 and hs-CRP in the same direction. Peripheral levels of IL-1β, IL-2, CXCL-8, IL-10, IFN-γ, MCP-1, MIP-1β and TNF-α were measured in at least 3 studies and, therefore, meta-analysed, but we did not find significant differences between FMS and HC (see **ESF2 Figures 1-10**). All analysis had significant heterogeneity ($I^2 > 50\%$).

Insert figure 2 around here

292 *3.3.2 Immune phenotypes*

293 The immune activation scores M1 and CIRS were significantly higher in FMS
294 patients than in HC, with medium effect size in both cases (g=0.31 and g=0.40, respectively).
295 No significant differences were found for Th-1, CMI or IRS (see **Table 1**). Noteworthy, we
296 removed two studies (Cordero et al., 2013; Malhotra et al., 2012) from the meta-analysis
297 reporting on IL-10, TNF-α, M1, CMI and CIRS because these data were considered to
298 constitute outliers.

299

300 *Insert table 1 around here*

301 *3.3.3 Sensitivity analysis*

302 In sensitivity analysis, the exclusion of some individual studies from the analysis did
303 alter the statistical significance (Hedges's g). As such, CXCL-8 was significantly higher in
304 FMS patients (g=0.33, 0.05 to 0.61, p=0.02) when one study was removed (Ribeiro et al.,
305 2018); TNF-α (g=0.30, 0.07 to 0.53, p=0.01) was higher in FMS patients after removing
306 another study (Hernandez et al., 2010) and hs-CRP (g=0.31, 0.05 to 0.57, p=0.02) after
307 removing one study (Ataoğlu et al., 2018). Moreover, removing one study (Hernandez et al.,
308 2010) from the IRS composite score changed the ES from non-significant to significantly
309 higher in FMS patients (g=0.20, 0.04 to 0.36, p=0.02). Removing one study did not change
310 the significance levels for IL-1β, IL-1RA, IL-2, IL-4, IL-17A, CXCL-11, IL-10, INF-
311 γ, MCP-1, MIP-1β, M1, Th-1, CMI or IRS.

312 *3.3.4 Publication bias and heterogeneity*

313 Publication bias was assessed with Egger's linear regression test (**Table 2**), which
314 revealed that none of the immune biomarkers presented significant publication bias.
315 Nevertheless, heterogeneity was very high I^2>75% or high I^2>50% for all biomarkers.
316 Egger's linear regression test revealed a potential publication bias for M1, and when using

Duval and Tweedie's trim and fill the imputed point estimate is 0.54 with 95% CI (0.29 to 0.80) for M1 to the right (favours FM).

Insert table 2 around here

3.4 Subgroup analyses

IL-6 levels were higher in FMS when considering the studies which a) excluded patients with MDD, b) reported that participants did not go through a medication washout, c) did not report the time of blood extraction, d) included study samples consisting of both sexes (**Table 3**), e) considered study samples with more than one year of difference in age between the study groups; and f) assessed the biomarkers with other assays than ELISAs (see **ESF1 Table 5**). CXCL-8 was higher in FMS patients when the participants did not have a medication washout and when the sample was mixed (**Table 3**).

Insert table 3 around here

Higher levels of M1 macrophage cytokines were found in FMS in studies reporting that patients with MDD were excluded to participate. Higher levels of M1, CMI and IRS phenotypes were found in FMS in studies reporting on medication washout or that the study groups comprised both men and woman. Higher levels of the M1 macrophage phenotype were found in FMS in studies that did not report the time of blood extraction while increased CIRS levels were found in studies which sampled blood in the morning hours.

Studies with a high number of red points in the checklist (>13.5) reported higher CIRS levels in the FMS group, whereas those with a lower score (<13.5) reported higher levels of M1 phenotype in the FMS group. CMI levels were higher in FMS patients when blood samples were analysed with other procedures than ELISA, but CIRS levels were found to be higher in FMS patients when the analyses where conducted with an ELISA procedure. M1 levels were higher in FMS patients when this group was more than one year older than

HC, but the opposite was found for CMI, which levels were higher in FMS when there were no differences in age between groups (see **ESF1 Table 6**). Subgroups of IL-10 and TNF-α were not applicable because studies were not equally distributed. Ethnicity and use of plasma versus serum did not have any significant effects on the immune biomarkers.

Post-hoc analyses were also performed using subgroups based on latitude (split in countries with higher vs lower of 40º) and the year when the study was conducted (before or after 2010) as explanatory variables. These analyses showed that IL-6, M1, CMI, CIRS levels were higher in studies performed in countries with lower latitude (<40º) and that IL-6 and M1 levels were higher in FMS in studies published after 2010 while CIRS levels were higher in FMS patients in studies published before 2010 (see **Tables ES5** and **ES6**).

3.5 Meta-regression

Meta-regression analyses showed that a significant part of the variance in the heterogeneity in IL-6 (11%), CXCL-8 (22%), IL-10 (38%), M1 (51%), CMI (41%), IRS (29%) and CIRS (16%) could be explained by confounders including sex, methodological quality of the studies and year of publication (**Table 4**).

Insert table 4 around here

4. Discussion

To our knowledge this is the largest systematic review, meta-analysis and meta-regression of immune biomarkers in FMS. Previous meta-analyses on immune biomarkers in FMS (Üçeyler et al., 2011) found that IL-6 was the only biomarker that was elevated in plasma in FMS, although these results were based on three studies only. The current more comprehensive meta-analysis provides results on IL-6 in 21 studies and meta-analyses ten more immune biomarkers. In addition, by calculating specific immune phenotypes, as delineated by the novel IRS/CIRS model, we were able to integrate essential but less well studied biomarkers including sIL-6R, CCL-11, IL-12, IL-7, IL-5, and IL-17A.

The first major finding of this meta-analysis is that IL-6, IL-17A, and IL-4 are significantly higher in FMS patients compared to HC. Nevertheless, there are no significant between-group differences in the majority of the immune biomarkers including IL-1β, IL-1RA, IL-2, CXCL-11, IL-10, hs-CRP, IFN-γ, MCP-1 and MIP-1β. Pro-inflammatory cytokines such as IL-6 or IL-17A are involved in the pathophysiology of pain (Zhang and An, 2007). Some authors posited that FMS symptoms may be caused by increased pro-inflammatory cytokine levels and decreased levels of anti-inflammatory cytokines (the inflammatory hypothesis) (Üçeyler et al., 2011; Zhang and An, 2007). However, our study shows that although some pro-inflammatory cytokines are slightly higher in FMS patients, the major anti-inflammatory cytokine IL-10 is not altered while IL-4 is even increased in FMS. IL-4 induces T0 cells to differentiate into immune-regulatory Th-2 cells thereby promoting the release of TGF-β, IL-1ra and IL-10 and suppressing the production of IL-1β, IL-6 and TNF-α (Maes and Carvalho, 2018). As such, the slightly increased levels of IL-4 in FMS patients could be explained as a compensatory response to increased levels of IL-6 and IL-17A (Maes and Carvalho, 2018; Pernambuco et al., 2013). Furthermore, IL-6 levels are difficult to interpret without measurements of sIL-6R and sgp130 which would allow to differentiate into pro-inflammatory IL-6 trans-signalling *versus* classical IL-6 signalling, which has homeostatic effects by increasing the production on sIL-1Ra and sTNFRs (Maes and Carvalho, 2018; Pernambuco et al., 2013). Thus, one of the main problems in FMS research is the interpretation of single cytokine assays (e.g. IL-6) while not considering their cytokine receptors, which may activate or suppress their activities (Maes and Carvalho, 2018; Pernambuco et al., 2013). Therefore, in the present study we meta-analysed multiple cytokines that were combined to reflect specific phenotypes, which allow to interpret possible disbalances between the major IRS and CIRS phenotypes (Maes and Carvalho, 2018).

The second major finding of this meta-analysis is that M1 macrophage, IRS and CIRS phenotypes are upregulated in patients with FMS as compared to controls. Nevertheless, our results do not reveal an inflammatory signature in FMS, since M1 is only slightly upregulated while there are no significant changes in hs-CRP or the Th-1 phenotype. Additionally, the between-group differences in M1 may be driven by IL-6 as this is the most assessed cytokine. As such, the findings of this meta-analysis do not confirm the "inflammatory hypothesis of FMS", which proposed that FMS is accompanied by increased M1 and IRS phenotypes and a downregulation of CIRS, Treg and Th-2 phenotypes (Bote et al., 2012; Ernberg et al., 2018; Pernambuco et al., 2013). The data of this meta-analysis indicate mild aberrations in the immune signature pointing toward a disrupted homeostasis with an upregulated M1/IRS and CIRS. IRS/CIRS disbalances are also reported in many neuro-psychiatric illnesses, including MDD or schizophrenia, but in those disorders the IRS and CIRS responses are much more pronounced than in FMS (Khandaker et al., 2015; Köhler et al., 2017; Maes and Carvalho, 2018).

In accordance with our a priori hypothesis, the results of the current study show a large heterogeneity in all immune biomarkers and immune phenotypes. For example, the use of MDD as an exclusion criterion may impact IL-6 and the M1 phenotype, suggesting that not excluding MDD patients may hamper the interpretation of IL-6 and M1 results in FMS. Moreover, the use of medication washout is another significant covariate which impacts IL-6 and CXCL-8 levels, as well as M1, CMI and IRS phenotypes. Thus, not only use of psychotropic drugs (Baumeister et al., 2016; Szałach et al., 2019) but also withdrawal of medication may affect the results. Not surprisingly, sex and age differences affected most biomarkers and immune phenotypes. Furthermore, reporting the time of blood extraction impacted the M1, CMI and IRS phenotypes. Latitude, 1990 versus 2010 ACR criteria, the type of assay used to analyse the biomarkers and our red points scale are other confounding

variables that affect the peripheral cytokines/chemokines levels and their phenotypes as well. Latitude may reflect the impact of environmental variables, such as hours of sun per day, pollution exposure or even diet. A plausible explanation for the effect of the 1990 versus 2010 ACR criteria is that by 2010 the conceptualization of FMS had evolved whereby the new ACR criteria included symptoms other than pain.

All in all, our results show that there are many different sources of variability that can induce heterogeneity in the levels of cytokines and chemokines and their phenotypes. A general conclusion is that not controlling for the confounders examined in our study may generate heterogeneity, which may lead to imprecise or even erroneous conclusions. Interestingly, in this meta-analysis we quantified the most important methodological errors that can induce heterogeneity and found that most studies were not adequately controlled for these background variables.

Interestingly, in chronic fatigue syndrome (CFS), which shows a strong comorbidity with FMS, there is evidence of immune activation with increased levels of CRP, and proinflammatory (TNF-α and IL-2) and immune-regulatory (e.g. IL-4) cytokines (Maes et al., 2019; Strawbridge et al., 2019; Yang et al., 2019). Nevertheless, the lack of a validated CFS case definition did not allow to use these immune biomarkers as external validating criteria for CFS (Maes et al., 2019; Yang et al., 2019). As such, there may be important differences in immune-inflammatory pathways between both disorders with signs of IRS and CIRS activation in CFS (Maes et al., 2019; Yang et al., 2019), whereas only minor changes could be established in FMS (this study).

The results of this meta-analysis should be discussed with regard to its limitations. First, the above-mentioned methodological differences and large heterogeneity among the studies made it difficult to draw solid conclusions. Although we were able to explain an important part of the heterogeneity in the results among studies, the percentage of

unexplained heterogeneity remained high. Other background variables may explain the residual heterogeneity including duration of illness, vitamin supplements, psychological stress, and a history of early trauma (Baumeister et al., 2016; Passos et al., 2015). In addition, some possibly important background variables could not be introduced in the meta-analysis because the number of studies was insufficient to perform the analysis, including the medications that the participants were taking in studies without a washout period (including PRN medications), the exact time of blood extraction, whether the participants had been fasting, substance abuse, smoking, the intake of omega 3 and antioxidant supplements, use of oral contraceptives, sedentarism, and cytokine data management when cytokine values were below the limit of detection. Future research should control for the many intervening variables listed in our comprehensive Immune Confounders Scale (ICS). Secondly, the small number of studies for some biomarkers including CXCL-11 and MIP-1β (3 studies), IL-4, IL-1ra, and IL-2 (4 studies). Therefore, we were unable to examine sub-group meta-analysis or meta-regressions for those cytokines/chemokines. Third, sensitivity analyses using the leave-one-out method showed that CXCL-8, TNF-α, M1 and CIRS had a significant effect size driven by a small number of studies. Fourth, the studies included herein reported results that were skewed to the assays of M1, CMI and T-reg phenotypes, whereas other phenotypes were underreported, including Th-1, Th-2, and Th-17. Fifth, only peer-reviewed articles were included, leaving out those un-published studies that were out of our knowledge and gray literature.

5. Conclusions

The results of this meta-analysis show that there may be a minor disbalance in the immune system in FMS with slightly upregulated IL-6, IL-4 and IL-17A levels as well as M1, CMI, IRS and CIRS phenotypes. Future research should control for the various background variables described in our study and should focus on a) IRS variables including

acute-phase reactants other than hs-CRP, such as haptoglobin, albumin or zinc; b) CIRS biomarkers including IL-4, IL-13, IL-5, and the T-reg phenotype; and c) classical IL-6 signalling versus IL-6 trans-signalling by measuring levels of IL-6 together with sIL-6R and sgp130 (Maes and Carvalho, 2018). Finally, a few larger-scale studies should examine a panel of 15-20 cytokines that would allow to estimate M1, Th-1, Th-2, Th-17, and T-reg phenotypes in the same studies.

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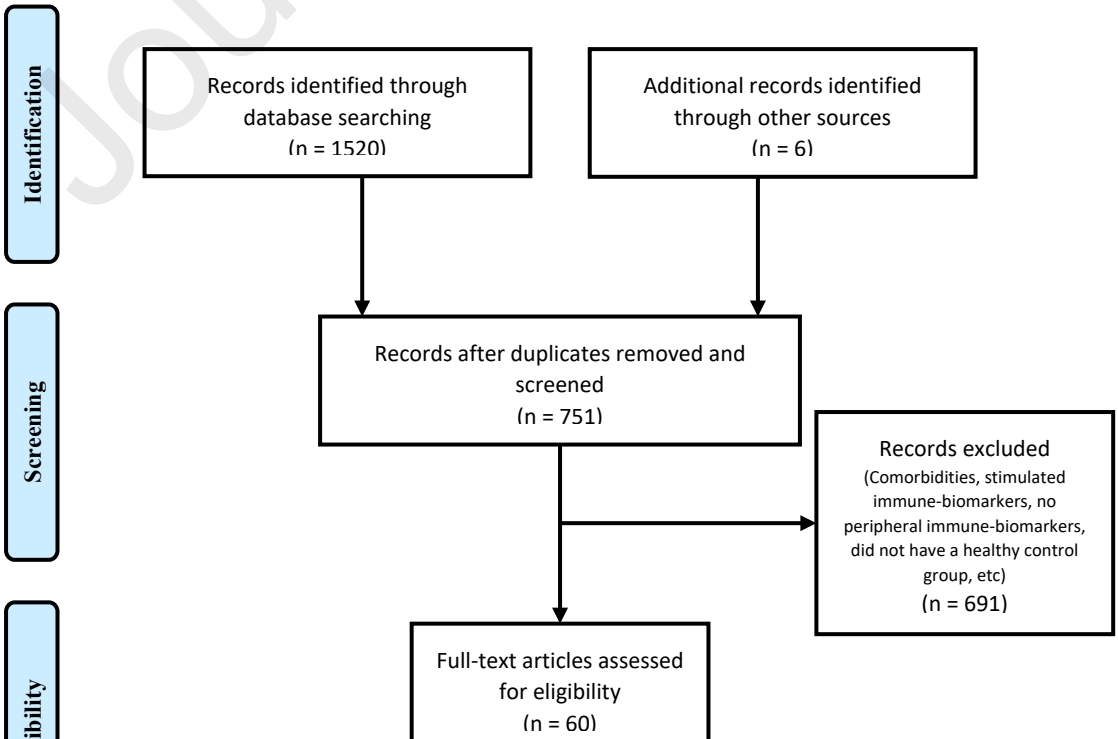
Figure 1. Flowchart of study selection



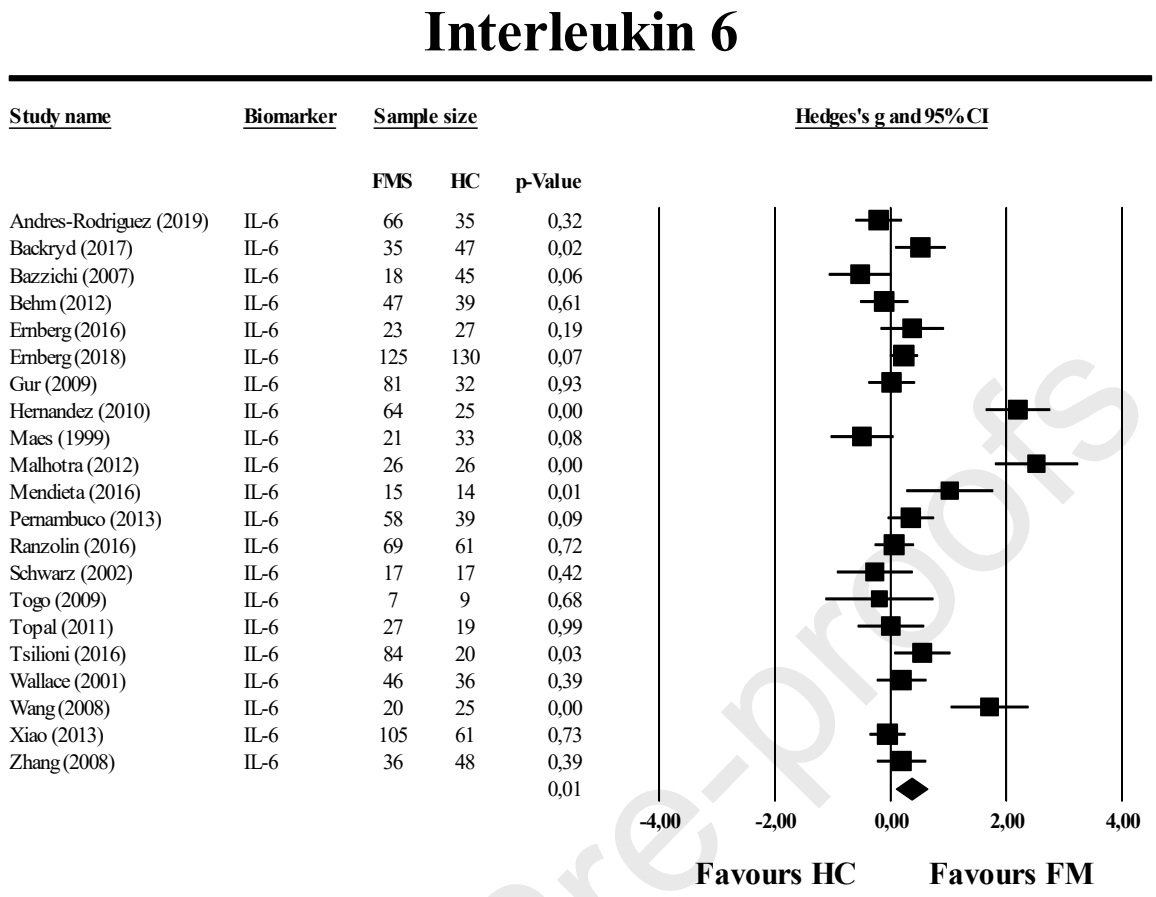


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Figure 2. Meta-analysis of interleukin 6

Highlights

1. Levels of IL-6, IL-4 and IL-17 are slightly upregulated in Fibromyalgia (FMS).
2. Immune-Inflammatory and the Compensatory (IRS/CIRS) phenotypes are also upregulated.
3. Research in immune-inflammatory in FMS needs to evaluate cofounding variables.
4. Immune-inflammatory studies in FMS need to focus on a wider spectrum of biomarkers.

Table 1. Meta-analysis and heterogeneity

Mediator	Studies	FMS	HC	Hedges' *g* (95% CI)	*p*-	*Heterogeneity*

	(n)	(n)	(n)		*value*	I^2	*p-value*
IL-1β	6	290	291	-0.08 (-0.37 to 0.21)	0.58	56.70	0.04
IL-1RA	4	228	247	0.44 (-0.01 to 0.90)	0.06†	80.40	0.002
IL-2	4	245	237	0.25 (-0.38 to 0.87)	0.44	89.45	<0.001
IL-4	4	245	237	0.50 (0.03 to 0.98)	**0.04***	81.87	0.001
IL-6	21	990	788	0.36 (0.09 to 0.63)	**0.01***	85.94	<0.001
IL-17A	4	254	264	0.53 (0.00 to 1.06)	**0.05***	87.15	<0.001
CXCL-8	15	767	685	0.19 (-0.14 to 0.51)	0.27	88.35	<0.001
CXCL-11	3	196	225	1.04 (-0.17 to 2.24)	0.09†	96.26	<0.001
IL-10^	9	468	473	0.17 (-0.16 to 0.50)	0.31	82.24	<0.001
hs-CRP	7	506	278	0.24 (-0.02 to 0.50)	0.07†	62.61	0.013
INF-γ	6	295	296	-0.09 (-0.90 to 0.73)	0.83	95.75	<0.001
MCP-1	6	420	399	0.03 (-0.38 to 0.43)	0.89	87.49	<0.001
MIP-1β	3	178	182	0.15 (-0.49 to 0.78)	0.65	87.81	<0.001
TNF-α^	12	610	562	0.06 (-0.35 to 0.47)	0.77	89.90	<0.001
M1^	23	1155	950	0.23 (0.03 to 0.43)	**0.02***	77.62	<0.001
Th-1	7	352	374	-0.12 (-0.74 to 0.50)	0.70	93.37	<0.001
CMI^	23	1147	948	0.14 (-0.04 to 0.31)	0.12	68.43	<0.001
IRS^	29	1474	1127	0.14 (-0.05 to 0.33)	0.15	78.43	<0.001

CIRS^	12	600	587	0.40 (0.09 to 0.72)	**0.01***	84.81	<0.001

Note: *$p < 0.05$, †$p < 0.10$ (marginally significant) ^ Removed one outlier (> 4 SD) *M1: IL-6, sIL-6R, TNF-α, IL-1β, IL-1RA, MCP-1 and MIP-1. Th-1: IL-2, IL-12, IFN-γ and sCD8. CMI: M1+Th-1. IRS: CMI, Chemokines (e.g. CXCL-8, CXCL-11), IL-7, IL-15, IL-17A, IL-18, IL-31, IL-33, HGMB1, and hs-CRP. CIRS: IL-4, IL-5, IL-13, sTNF-α R1, sTNF-α R2, IL-1RA, sgp130, and IL-10.* hs-CRP= high sensibility C-reactive protein; IFN-γ= Interferon-gamma, MCP-1= Monocyte chemoattractant protein-1; TNF-α= Tumor necrosis factor alpha; M1= Macrophage; Th-1: T helper-1; CMI= Cell Mediated Immunity; IRS= Immune-inflammatory Response System; CIRS= Compensatory Immune-Regulatory Reflex System.

Table 2. Publication bias

Mediator	Studies (n)	*t*	*df*	*p-value (Egger)*	*Fail-Safe N*
IL-1b	6	0.09	4	0.93	0
IL-1ra	4	1.95	4	0.19	12
IL-2	4	0.47	2	0.68	7

IL-4	4	1.20	2	0.35	19
IL-6	21	1.57	19	0.13	168
IL-17A	4	1.05	2	0.40	24
CXCL-8	15	0.32	13	0.75	21
CXCL-11	3	2.21	1	0.27	36
IL-10^	9	0.84	7	0.43	1
hs-CRP	7	0.61	5	0.57	11
INF-γ	6	0.57	4	0.60	0
MCP-1	6	0.71	4	0.51	0
MIP-1β	3	0.97	1	0.51	0
TNF-α^	12	0.52	10	0.61	0
M1^	23	2.04	21	0.05*	79
Th-1	7	1.14	5	0.30	0
CMI^	23	0.69	21	0.49	16
IRS ^	29	0.21	27	0.83	40
CIRS^	12	1.51	10	0.16	85

643 Note: *p < 0.05, †p < 0.10 (marginally significant) ^ Removed one outlier (> 4 SD). *M1: IL-6, sIL-6R,*

644 *TNF-α, IL-1β, IL-1RA, MCP-1 and MIP-1. Th-1: IL-2, IL-12, IFN-γ and sCD8. CMI: M1+Th-1. IRS: CMI,*

645 *Chemokines (e.g. CXCL-8, CXCL-11), IL-7, IL-15, IL-17A, IL-18, IL-31, IL-33, HGMB1, and hs-CRP. CIRS:*

646 *IL-4, IL-5, IL-13, sTNF-α R1, sTNF-α R2, IL-1RA, sgp130, and IL-10.* hs-CRP= high sensibility C-reactive

protein; IFN-γ= Interferon-gamma, MCP-1= Monocyte chemoattractant protein-1; TNF-α= Tumor necrosis factor alpha; M1= Macrophage; Th-1: T helper-1; CMI= Cell Mediated Immunity; IRS= Immune-inflammatory Response System; CIRS= Compensatory Immune-Regulatory Reflex System.

Table 3. Meta-analysis by groups

Variable	Meta-analysis					Heterogeneity	
	n	Hedges' *g*	SE	*p*-value	*Total between p-value*	I^2	*p*-value
IL-6							
Reported exclusion of MDD					0.27		
No	11	0.20	0.14	0.16		75.12	<0.001

Yes	10	0.52	0.25	**0.04***		90.44	<0.001
Reported Medication					0.21		
Washout							
Yes	8	0.12	0.25	0.64		87.05	<0.001
No	13	0.50	0.16	**0.002***		84.32	<0.001
Time of extraction					0.22		
Morning	11	0.26	0.21	0.21		89.19	<0.001
NR	9	0.53	0.23	**0.02***		82.56	<0.001
Sex					0.25		
100% woman	10	0.23	0.21	0.28		85.86	<0.001
Mixed	10	0.53	0.21	**0.012***		88.20	<0.001
CXCL-8							
Reported exclusion					0.29		
of MDD							
No	10	0.05	0.20	0.80		87.37	<0.001
Yes	5	0.46	0.33	0.16		89.68	<0.001
Reported Medication					0.08		
Washout							
Yes	6	-0.25	0.33	0.40		90.41	<0.001
No	9	0.43	0.20	**0.03***		87.62	<0.001

Time of extraction					0.53		
Morning	8	0.04	0.26	0.87		92.91	<0.001
NR	6	0.38	0.29	0.19		76.25	0.001
Sex					**0.05***		
100% woman	9	-0.15	0.23	0.51		84.96	<0.001
Mixed	5	0.74	0.30	**0.015***		92.90	<0.001

Note: *$p < 0.05$, †$p < 0.10$ (marginally significant)

Table 4. Meta-regression

Mediator	Studies (n)	2-sided p-value	R analog
IL-6	20		
Total females		0.005	0.11
CXCL-8	13		
Total females		0.001	0.22
IL-10	10		

Year of publication		0.0036	0.38
M1	24		
Total females		<0.001	
			0.51
Methodological quality		0.06	
CMI	22		
Methodological quality		<0.001	0.41
IRS	27		
Methodological quality		<0.001	0.29
CIRS	12		
Total females		0.001	0.16

Note: *$p < 0.05$, †$p < 0.10$ (marginally significant) ^ Removed one outlier (> 4 SD). *M1: IL-6, sIL-6R, TNF-α, IL-1β, IL-1RA, MCP-1 and MIP-1. CMI: M1+Th-1. IRS: CMI, Chemokines (e.g. CXCL-8, CXCL-11), IL-7, IL-15, IL-17A, IL-18, IL-31, IL-33, HGMB1, and hs-CRP. CIRS: IL-4, IL-5, IL-13, sTNF-α R1, sTNF-α R2, IL-1RA, sgp130, and IL-10.* M1= Macrophage; Th-1: T helper-1; CMI= Cell Mediated Immunity; IRS= Immune-inflammatory Response System; CIRS= Compensatory Immune-Regulatory Reflex System.