Original experimental

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Exercise-induce hyperalgesia, complement system and elastase activation in Myalgic Encephalomyelitis/Chronic Fatigue Syndrome – a secondary analysis of experimental comparative studies

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Abstract

Background and aims: The interaction between the immune system and pain has been thoroughly explored in the recent decades. The release of inflammatory mediators from immune cells has the capability of activating neurons and glial cells, in turn sensitizing the nervous system. Both immune system alterations and pain modulation dysfunctions have been shown in myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) following exercise. However, no studies tried to explore whether these two phenomena are linked and can explain exercise-induced symptoms worsening in people with ME/CFS. We hypothesized that exercise-induced changes in descending pain modulation is associated to changes in immune system functions. We used complement system

product C4a and elastase activity as indicators of immune system activity.

Methods: The study design was a secondary analysis of controlled experimental studies. Twenty-two patients with ME/CFS and 22 healthy sedentary controls were enrolled. In experiment 1, subjects performed an aerobic submaximal exercise test; in experiment 2 they underwent a self-paced exercise test. One week of rest period were set between the two exercise tests. Before and after each experiment, subjects underwent clinical assessment, pain thresholds (PPTs) measurement, and blood sampling. Immune system function was assessed measuring complement system C4a products and elastase activity.

Results: Changes in elastase activity were not associated to changes in PPTs. Associations were observed in the ME/CFS group between changes in PPTs and C4a products, following both types of exercise. After submaximal exercise, the change in C4a products was associated with the change in PPT at the thumb in patients ($r=0.669$, $p=0.001$). Similarly, after self-paced exercise the change in C4a products was associated with the change in PPT at the calf in patients ($r=0.429$, $p=0.047$). No such correlations were found in healthy controls. Regression analysis showed that C4a changes after the submaximal exercise significantly predicted the change in PPTs ($R^2=0.236$; $p=0.02$).

Conclusions: Moderate associations between exercise-induced changes in PPTs and immune system activity were found only in ME/CFS. The change in the complement system following submaximal exercise might be able to explain part of the change in patient's pain thresholds, providing evidence for a potential link between immune system alteration and dysfunctional endogenous pain modulation. These results have to be taken with caution, as only one out of three measures of PPTs was found associated with C4a changes. We cannot reject the hypothesis that C4a might therefore be a confounding factor, and changes during exercise might be mediated by other mechanism.

Implications: Immune system changes following exercise might contribute to exercise-induced symptoms

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worsening in patients with ME/CFS. However, the role of the complement system is questionable.

Keywords: pain; immune system; exercise; chronic fatigue syndrome; pain threshold; hyperalgesia.

1 Introduction

The immune system has been extensively shown as capable of modulating nociceptive inputs and central nervous system processes (reviewed in [1] and [2]). Immune cells and glia interact with peripheral and central neurons through the release of inflammatory mediators, that in turn alter nociceptive processing and lead to hyperalgesia in animals [3–6]. Patients with chronic pain might show altered levels of immune system markers such as pro-inflammatory cytokines and other inflammatory markers [7, 8]. Inflammatory markers might rapidly decrease after a single bout of aerobic, sub-maximal exercise [9]. However, it is unclear whether the reduction of immune markers determines an improvement in symptoms in humans. In addition, this might be different for other conditions, like myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS). Two systematic reviews showed that cytokine expression do not differ between people with ME/CFS and healthy controls at baseline [10, 11] or following exercise [11, 12]. However, exercised-induced changes in other immune markers such as toll-like receptor 4 (TLR4), and products of the complement system have been reported [12]. A better understanding of the relation between the immune system and nociceptive modulation would allow the development of new analgesic treatments, improving the management of different chronic pain conditions.

The complement system (CS) is part of both innate and adaptive immunity [13, 14]. It includes several proteins that interact with each other through complex regulatory mechanisms at both RNA and protein levels [15]. The CS plays a role in immune response enhancement, immune complexes clearance and even synaptic maturation and angiogenesis [14], and it has been proposed as key mediator in pain states. After an injury, complement fragments C3a and C5a are produced and they are able to recruit and activate immune cells [14] – in turn enhancing inflammatory response. C3a and C5a injection in an otherwise healthy tissue induced nociceptive sensitization, hyperalgesia to heat, and neuroinflammation, in animals [16, 17].

Similarly, other pathways can potentially play a role in inducing nociceptive sensitization and inflammation. A relevant one involves the activity the enzyme elastase.

This enzyme exerts proteolytic cleavage of a number of structurally and functionally vital proteins, including protease-activated receptor-2 (PAR₂) [18] and Ribonuclease L (RNase L) [19]. PAR₂ in turn activates transient receptor potential channels of nociceptive fibers, leading to neurogenic inflammation and hyperalgesia [18]. RNase L activation is associated with protein kinase R (PKR) activation and nucleus transcription factor κ B (NF- κ B) expression [20, 21]. NF- κ B has a recognized role in both up-regulating genes responsible for immune responses and in nervous system plasticity [22, 23]. Taken together, these findings support the role of elastase in initiating a downstream cascade of events that ultimately leads to neuronal sensitization, immune activation, and hyperalgesia. Elastase has indeed been shown to cover a role in the pathophysiology of chronic inflammation and rheumatoid arthritis [24, 25].

In humans, CS and elastase activations in relation to pain have been studied in people with ME/CFS. ME/CFS is a condition characterized by many symptoms, being post-exertional malaise, fatigue and pain the most disabling ones [26]. In addition, patients with ME/CFS show a malfunctioning descending modulatory system for nociceptive stimuli [27]. Following aerobic exercise, healthy people normally show an increase in pressure pain thresholds (PPTs) [28] – a phenomenon known as exercise-induced hypoalgesia (EIH). On the contrary, the same exercise fails to induce hypoalgesia in patients with ME/CFS, and PPTs might even decrease following exercise [27–30]. This accounts for dysfunctional EIH in patients with ME/CFS. Exercise or physical activity indeed induce symptoms worsening in ME/CFS patients that can last up to 24–48 h [26].

Evidence from animal and human studies suggest that immune system activation might contribute to both the pathophysiology of ME/CFS and the etiology of post-exertional malaise [7, 12, 31–33]. Sorensen et al. [34] studied three key products of the CS, namely C3a, C4a, and C5a, and found that only C4a increased in ME/CFS patients following exercise. This result is in line two previous studies from our group, showing a change in C4a levels and a decrease in PPTs in ME/CFS patients following exercise [27, 35]. Together with C4a changes, exercise-induced elastase alterations in ME/CFS patients have been reported, too [36, 37]. However, to the best of our knowledge no studies have investigated whether this exercise-induced immune system alteration can be linked to dysfunctional EIH in any chronic pain population.

The aim of the present work was therefore to investigate the link between dysfunctional EIH and immune system responses. To answer to our research question, we

performed a secondary analysis of original data from our previous works [27, 35]. C4a products and elastase activity were used as measures of the immune function. PPTs are a reliable [38] and widely used tool for assessing local or widespread hyperalgesia [39–41]. We hypothesized that changes in C4a products and elastase activity following exercise are associated to hyperalgesia in ME/CFS patients.

2 Methods

2.1 Subjects

Patients with diagnosis of ME/CFS were recruited from a private clinic specialized in internal medicine. Subjects had to fulfil eligibility criteria as described by the Centre for Disease Control and Prevention Criteria for ME/CFS [42]. Definite diagnosis of ME/CFS could be made only if the patient suffered from severe fatigue for at least 6 months which interfered with their daily and/or work-related activities, and if other possible pathological conditions which could explain the symptoms had been ruled out. In addition, patients needed to experience at least four out of the following eight symptoms: (a) post-exertional malaise lasting more than 24 h; (b) unrefreshing sleep; (c) significant impairment of short-term memory or concentration; (d) muscle pain; (e) multi-joint pain without swelling or redness; (f) headaches of a new type, pattern, or severity; (g) tender cervical or axillary lymph nodes; (h) frequent or recurring sore throat. Given that pain covers an important role in ME/CFS, and that the aim of the study was related to hyperalgesia, patients were only included if they also presented chronic widespread pain as defined by the American College of Rheumatology criteria for fibromyalgia [43].

The control group was selected from relatives or friends of people involved in the research project and, in some cases, from patient's relatives or friends. In order to minimize possible genetic influences to the phenomena under study, no patient's close relatives were enrolled. Inclusion criteria for the control group were to be healthy and to report no pain. Given the influence that physical activity is known to exert on general health and pain symptoms, we only include healthy controls that reported to have a sedentary life-style [44, 45]. Subjects were defined as sedentary in case they had a seated occupation and did not perform more than 1 h of sport per week.

All subjects needed to be between 18 and 65 years of age. We only included women, as sex has been shown to be an important source of bias in ME/CFS. Pain sensitivity differs between men and women, and predominantly women suffer ME/CFS [46, 47].

2.2 Procedure

The Ethics Committee of our University Hospital approved the study protocol. All subjects were well informed about the aim of the study and the procedures and provided written informed consent before data collection was initiated. Subjects visited the university 3 times for data collection.

During the first visit (day 1), personal and demographic characteristics were collected and subjects their height and weight was measured. Subjects were asked to refrain from taking analgesic medication from that point on.

The second visit took place (day 7) 1 week after the first visit. Subjects underwent an extensive clinical examination for the assessment of ME/CFS symptoms and other health-related features [27, 35]. An experienced nurse collected four venous blood samples, for a total of 32.5 cc. Samples were coded and brought to the biology lab of UZ Brussel within 1 h from sampling, where they were processed (centrifuging at 1500 g for 10 min, to separate whole blood cells from plasma and serum) and stored at -80°C until needed for the analysis. Blood samples were then sent to RED Laboratories N.V. (Zellik, Belgium) for the analysis of immune variables. Hence, the laboratory did not know the identity or the healthy status of the subject from which the samples were taken. PPTs were measured by an assessor who was blinded to the health status (healthy control or ME/CFS) of the subject. Next, study participants performed a submaximal stress test on an electrically braked cyclo-ergometer (Excalibur Lode, Groningen, the Netherlands). PPTs were reassessed immediately post-exercise and 1 h after completing the exercise. Measures and exercise testing were performed in a quiet room with a controlled temperature of $18\text{--}20^{\circ}\text{C}$.

One week after the second visit, the third visit (day 14) took place. Subjects underwent the same exact procedure, but instead of the submaximal exercise test they performed a self-paced and physiologically limited exercise test.

Further details regarding setting and procedures can be found in our previously published works [27, 35].

2.3 Outcome measures

2.3.1 Pain pressure thresholds

PPTs were measured using an analog pressure algometer (Force Dial models FDK 10 and FDK 40 Push Pull Force Gate, Wagner Instruments, Greenwich, CT, USA) at three different sites: (1) in the interdigital web skin between the thumb and the index finger, (2) 5 cm laterally to L3 spinous process, (3) at the proximal third of the calf muscle. The test order of the sites was randomized by lottery and three consecutive measures with an in-between interval of ≥ 10 s were performed per site. The PPT was calculated as the mean of the last two measurements. Pressure algometry has been showed to be a reliable measure of PPTs [38].

2.3.2 Determination of C4a products

To determine the levels of C4a the BD OptEIA™ ELISA kit (BD Biosciences, CA, USA), which utilizes a specific C4a/C4a-desArg antibody for *in vitro* analysis of human plasma, was used. The assay has a minimal detectable dose of 0.006 ng mL^{-1} , limited cross reactivity, and adequate intra- and inter-assay precision (% coefficient of variation ranges from 4.0 to 6.5 and from 6.5 to 9.7, respectively). The procedure is described in detail in our previous work [35]. The reader can also refer to the manufacturer's manual for further details about the procedure, reagent preparation and specimen collection and handling.

2.3.3 Determination of elastase activity

To determine elastase activity, we used EnzChek® Elastase Assay Kit E-12056 (Molecular Probes, OR, USA). Details on the exact procedure and materials used for elastase measuring can be found elsewhere [36]. Since reliability and validity of the assay are not available in literature, we double-checked the results by randomly picking 15 samples up and re-analysing them using the ELISA elastase kit RD191021100 (BioVendor GmbH, Germany). Both assays gave almost identical results.

2.4 Exercise tests

2.4.1 Submaximal exercise test

The submaximal exercise test which was performed, is known as the aerobic power index [48] and has been

found reliable for the examination of both healthy inactive people as patients with ME/CFS (ICC coefficient: 0.98 and 0.97, respectively) [48, 49]. Participants were asked to maintain a cycling rate at 60–70 rotations per minute while the increased by 25 watts per minute until 75% of the age-predicted target heart rate was reached [50]. If subjects were unable to reach their individual target heart rate, the workload achieved during the last minute of exercise was recorded as the final power output.

2.4.2 Self-paced and physiologically limited exercise test

The same workload and cycling rate as during the submaximal exercise test was used but based on the pacing principles. This means that three safety breaks were used to manipulate the exercise in order to avoid possible over-exertion in ME/CFS [37]. The first safety break limited the heart rate, which could not exceed 80% of the heart rate which corresponded with the anaerobic threshold during the submaximal exercise test. When the anaerobic threshold could not be reached, the highest achieved heart rate was used. If the heart rate during the self-paced exercise reached this predetermined threshold, the workload was decreased. If this did not suffice, cycling rate was decreased, too. The second safety break limited the maximum workload and was set at the 80% of the anaerobic threshold during the submaximal test. The third safety break limited the exercise duration. In order to pace the exercise appropriately, subjects were asked to estimate their current physical capabilities prior to commencing the activity, keeping in mind the regular fluctuating nature of their symptoms [37]. Thus, subjects were asked to estimate how long they thought they would have been able to cycle without exacerbating their symptoms. To further reduce the risk of overestimating their condition, test duration was limited to 75% of the estimated time if subjects reported to have a “good day” [37] and to 50% of the estimated time when subjects reported to have a “bad day” [51]. This method was used to limit the exercise duration in ME/CFS patients, as well as in healthy controls.

2.5 Statistical analysis

Data were analysed using SPSS 23.0 (SPSS, Inc., Chicago, IL, USA). Given the small samples, and the use of indirect measures, we performed more conservative non-parametric Mann-Whitney *U*-tests to compare baseline values between groups.

Non-parametric correlation analysis was performed to analyse the association between immune factors and PPTs at baseline and after exercise. Then we performed correlation analysis to assess the association between changes of C4a products and elastase activity and changes of PPTs after both types of exercise in both patients and healthy controls. Linear regression analysis was also performed after testing assumptions. If assumptions were not met, Weighted Least Squared (WLS) regression were used.

3 Results

Twenty-two women with ME/CFS and 22 healthy sedentary women participated in the study. Mean age ($\pm$ SD) of the CFS group was 34.3 ± 8.8 years and of the healthy controls was 38.9 ± 15 years. Mean BMI in the CFS group was 24.1 ± 4.7 kg*m² and in the healthy control group it was

24.5 ± 4.8 kg*m². Between group analysis at baseline showed no differences for age and BMI ($p=0.341$ and 0.814 , respectively). In baseline (visit 1), 10 patients in the ME/CFS group reported the use of anti-depressants, and 12 used analgesics. One subject in the control group reported using anti-depressants.

The results of the between group comparison and/or the between exercise tests comparison regarding accelerometry, exercise response, exercise capacity, PPTs, elastase activity, and complement C4a levels have been reported elsewhere [5, 10]. Here, we report within-group and between-group comparisons regarding PPTs, elastase activity, and complement C4a levels during either sub-maximal exercise test or self-paced/physiologically-limited one, in Table 1 and Table 2, respectively.

The current analysis showed that PPTs measured prior to and following the exercise tests were not significantly correlated to elastase activity or C4a products in either group (ME/CFS patients or healthy controls). In addition,

Table 1: Pressure pain thresholds (PPTs), elastase levels and C4a products pre- and post-submaximal exercise (experiment 1) in myalgic encephalomyelitis/chronic fatigue syndrome (CFS) patients and healthy controls.

	ME/CFS				Healthy controls				U
	Pre [median (IQR)]	Post [median (IQR)]	Changes [median (IQR)]	W	Pre [median (IQR)]	Post [median (IQR)]	Changes [median (IQR)]	W	
PPTs (kg/cm ²)									
Thumb	4.22 (2.33)	3.90 (1.93)	-0.12 (1.35)	0.626	4.50 (2.80)	4.85 (2.62)	0.23 (1.38)	0.058	0.084
Back	4.25 (1.90)	4.15 (2.28)	0.02 (1.16)	0.795	5.75 (2.96)	6.70 (4.17)	0.80 (1.51)	0.002 ^b	0.009 ^a
Calf	4.54 (1.63)	4.60 (2.07)	0.21 (1.58)	0.783	5.17 (1.55)	6.36 (2.71)	0.68 (1.10)	0.000 ^b	0.018 ^a
Immune factors									
Elastase	60.00 (100.35)	54.00 (68.9)	-3.60 (66.25)	0.881	68.40 (38.75)	56.00 (37.27)	-2.00 (24.50)	0.602	0.894
C4a	2.99 (0.6)	2.73 (0.65)	-0.25 (0.39)	0.002 ^b	3.00 (0.52)	2.47 (0.81)	-0.29 (0.76)	0.006 ^a	0.526

IQR=interquartile range; W=Wilcoxon Signed Ranks test for within group analysis; U=Mann-Whitney U for between groups comparisons of changes. ^aStatistically significant difference for p -value below 0.05; ^bstatistically significant difference for p -value below 0.01.

Table 2: Pressure pain thresholds, elastase levels and C4a products pre- and post-self-paced/physiologically-limited exercise (experiment 2) in myalgic encephalomyelitis/chronic fatigue syndrome (CFS) patients and healthy controls.

	ME/CFS				Healthy controls				U
	Pre [median (IQR)]	Post [median (IQR)]	Changes [median (IQR)]	W	Pre [median (IQR)]	Post [median (IQR)]	Changes [median (IQR)]	W	
PPTs (kg/cm ²)									
Thumb	4.20 (1.78)	3.63 (1.82)	-0.31 (0.64)	0.000 ^b	4.52 (2.60)	4.82 (3.29)	0.13 (0.61)	0.399	0.001 ^b
Back	4.14 (2.88)	4.70 (2.90)	0.06 (1.08)	0.485	5.29 (3.47)	6.36 (3.92)	1.21 (1.28)	0.000 ^b	0.001 ^b
Calf	5.05 (2.64)	4.91 (2.21)	-0.15 (1.00)	0.404	5.05 (2.62)	5.95 (2.62)	0.48 (1.09)	0.013 ^a	0.007 ^b
Immune factors									
Elastase	65.40 (81.60)	58.80 (57.2)	2.05 (78.88)	0.808	60.60 (61.45)	35.50 (28.40)	-6.30 (53.15)	0.058	0.190
C4a	2.87 (0.50)	2.54 (0.63)	-0.25 (0.44)	0.004 ^b	3.13 (0.47)	2.86 (0.88)	-0.29 (0.62)	0.002 ^b	0.999

IQR=interquartile range; W=Wilcoxon Signed Ranks test for within group analysis; U=Mann-Whitney U for between groups comparisons of changes. ^aStatistically significant difference for p -value below 0.05; ^bstatistically significant difference for p -value below 0.01.

Table 3: Associations between changes of pressure pain thresholds (PPT) and changes of immune factors (C4a products) in healthy controls and patients with myalgic encephalomyelitis/chronic fatigue syndrome (CFS).

Type of exercise	Immune factor	PPTs	r	SR
ME/CFS				
Submaximal	C4a	Thumb	+0.669	0.001 ^b
	C4a	Back	+0.234	0.294
	C4a	Calf	+0.266	0.232
Self-paced	C4a	Thumb	+0.164	0.453
	C4a	Back	-0.158	0.484
	C4a	Calf	+0.429	0.047 ^a
HC				
Sub-maximal	C4a	Thumb	-0.080	0.725
	C4a	Back	+0.273	0.219
	C4a	Calf	-0.057	0.801
Self-paced	C4a	Thumb	+0.273	0.218
	C4a	Back	-0.023	0.921
	C4a	Calf	+0.134	0.553

Only correlations between C4a products and PPTs are reported. Elastase activity did not show significant correlation in either the CFS group nor healthy controls. r = correlation coefficient; SR = Spearman's Rho for non-parametric correlations. ^aStatistically significant difference at SR, for *p*-value below 0.05; ^bstatistically significant difference at SR, for *p*-value below 0.01.

changes in elastase activity were not associated to changes in PPTs in neither of the exercise bouts. However, when analysing possible associations between changes in PPTs and complement products, significant correlations were observed after both exercise bouts. After the submaximal exercise test, the reduction of PPTs at the thumb showed a very strong correlation with the change in C4a ($r = 0.669$; $p = 0.001$ – see Table 3 and Fig. 1 for details). In addition, after the paced exercise, the decrease in PPTs at the calf was correlated with the change in C4a ($r = 0.429$; $p = 0.047$, Table 3). The remaining correlations were non-significant. Regression analysis showed that the change in C4a following experiment 1 significantly predicted the change of PPTs at the thumb ($R^2 = 0.238$; $p = 0.02$. Model fit: $F = 6.25$; $p = 0.2$). WLS regression analysing the relation between changes in C4a and PPTs at the calf, was not significant.

On the contrary, we found no correlations in healthy controls (Fig. 1 and Table 3).

4 Discussion

We investigated whether immune system changes following exercise can be associated to a common feature of

ME/CFS patients – that is the dysfunction in the endogenous pain inhibition. To the best of our knowledge, this is the first attempt to explore this association in ME/CFS or any other chronic pain conditions.

Our group previously showed that in healthy inactive people pain pressure thresholds increase in response to exercise [27]. On the contrary, PPTs in people with ME/CFS decreased following exercise [27]. These responses were observed following a submaximal exercise, as well as a self-paced and physiologically-limited exercise. Furthermore, the decrease in PPTs was shown to be associated with post-exertional malaise and symptom exacerbation in ME/CFS [27]. In addition, post-exertional increase in pain and fatigue were related to two immune factors, namely C4a products and elastase, respectively [35]. However, it had not previously been explored whether immune system changes and descending pain inhibitory activity in response to exercise are related.

While no association between elastase activation and pain was found, both ME/CFS patients and healthy subjects showed a similar decrease of C4a products in response to exercise. However, no relation was found between the change in complement products and the changes in pain thresholds in healthy controls. On the contrary, exercise-induced C4a changes seen in patients were associated with pressure pain thresholds changes. Interestingly, complement activity was not associated with pain thresholds at rest, or after the exercises, but it is rather the magnitude of exercise-induced changes that showed association. This is important, as post-exertional symptoms worsening is a characteristic peculiar of people with ME/CFS [52]. Fatigue and pain do not worsen after sub-maximal exercise in other conditions affected by fatigue such as depression and multiple sclerosis [53–55].

The CS has a key role in innate immune responses as well as in humoral immunity, and its activation ultimately promotes inflammatory and neuro-inflammatory responses [56]. CS activation has been related to hyperalgesia in both inflammatory and neuropathic animal pain models [57–59]. These studies showed that the increased amount of complement proteins C3a and C5a can induce hyperalgesia via both peripheral and central (i.e. microglia-mediated) mechanisms. However, only C4a, and not C3a or C5a, changed after exercise in ME/CFS patients [34].

To the best of our knowledge, C4a has not been previously related to pain. However, a number of autoimmune diseases, like Systemic Lupus Erythematosus, Rheumatoid Arthritis, or autoimmune hepatitis, have been associated with C4a suppression or C4 gene depletion [60–63]. C4a deficiencies was associated with elevated level of immune

Fig. 1: Associations between exercise-induced complement system changes and pain threshold changes in healthy controls and patients with chronic fatigue syndrome. No significant correlations have been found in healthy controls. Details of the correlations can be found in Table 3.

complexes [64]. These findings corroborate the hypothesis that a suppression in the CS might be associated with over-reaction of autoimmune pathogens, which in turn is capable to explain ME/CFS patients' symptoms after exercise or physical activity [65–67]. This hypothesis is in line with our current understanding of the great complexity of the immune system, which shows a great amount of interplay within the immune system itself, but also with other systems namely the endocrine and nervous system [68]. Complex, reciprocal, and far from being understood interactions show that the immune response is not straightforward, but it often includes a combination of enhanced and suppressed responses.

Interestingly, the majority of immune factors described in the scientific literature increases immediately

post-exercise in ME/CFS [12]. In two similar studies, C4a was found to increase after sub-maximal exercise [32, 34], but this was 6 h after exercise, and no actual changes were found immediately after exercise. Here we have found a quick suppression of C4a products, suggesting that C4a is initially suppressed, and then increases over time to eventually peak a few hours after exercise. The change in C4a can explain 24% of the change in pain thresholds.

Notably, we found interesting results although we used indirect measures to assess a systemic immune response and pain modulatory mechanisms. In addition, exercises were tailored to the patient's ability, in a safe and supervised environment, and both tests were stopped as soon as patients reached the target heart rate, or when they chose to stop. Both types of exercise

test – especially the self-paced one – were chosen in order to induce a limited amount of stress or no stress at all and avoid severe symptom exacerbations. Despite this, associations between C4a changes and pain changes following exercise were found even after a low-demanding, self-paced exercise. A quick suppression of C4a might be part of the immune response associated with exercise-induced hyperalgesia in ME/CFS patients.

However, our results need to be interpreted with caution. First, sample size was small, and associations between changes in complement system activation were associated to changes in pain thresholds in only one out of three body areas in both exercises. For these reasons, it cannot be completely excluded that our findings are just coincidental. Alternatively, C4a might be a confounding factor, and its change after a single bout of exercise might be mediated by other mechanisms. Other immune factors, inflammatory markers or oxidative stress might be more important contributors to the dysfunctional endogenous pain inhibition that ME/CFS patients display during exercise and are worth exploring. Anti-oxidant response after exercise is delayed and reduced [10, 12]. Changes in gene expression following exercise can also account for ME/CFS symptoms. In particular, interleukin-10 and Toll-like receptor 4 gene expression increase in ME/CFS patients but not in healthy people [33, 69].

5 Conclusion

Not all the changes in PPTs in response to exercise were related to changes in immune factors. However, strong and moderate associations between the exercise-induced changes in PPTs measured at the hand and the leg and complement protein C4a were found in CFS, but not in the healthy inactive people. We acknowledge that complement protein C4a decreased in both ME/CFS patients as in healthy people, but this decrease was associated with the changes in the PPTs only in the ME/CFS group. Despite some limitations, our findings support the hypothesis that immune system responses can play a role in, at least partially, unravelling those mechanisms that are involved in the dysfunctional endogenous modulation seen in ME/CFS. Further investigation is warranted and should include CS products but also other immune or inflammatory mediators. Finally, we highlight the importance of assessing exercise-induced changes, and not only to absolute values, when dealing with ME/CFS patients.

Authors' statements

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Informed consent: All subjects were well informed about the aim of the study and the procedures and provided written informed consent before data collection was initiated.

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