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Suppressed immune and metabolic responses to intestinal damage-associated microbial translocation in myalgic encephalomyelitis/chronic fatigue syndrome

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ABSTRACT

The etiology and mechanism of myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) are poorly understood and no biomarkers have been established. Specifically, the relationship between the immunologic, metabolic, and gastrointestinal abnormalities associated with ME/CFS and their relevance to established symptoms of the condition remain unclear. Relying on data from two independent pairs of ME/CFS and control cohorts, one at rest and one undergoing an exercise challenge, we identify a state of suppressed acute-phase innate immune response to microbial translocation in conjunction with a compromised gut epithelium in ME/CFS. This immunosuppression, along with observed enhancement of compensatory antibody responses to counter the microbial translocation, was associated with and may be mediated by alterations in glucose and citrate metabolism and an IL-10 immunoregulatory response. Our findings provide novel insights into mechanistic pathways, biomarkers, and potential therapeutic targets in ME/CFS, including in the context of exertion, with relevance to both intestinal and extra-intestinal symptoms.

1. Introduction

Myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) is a complex condition characterized by extreme fatigue, as well as problems with cognition, sleep, and pain, that do not improve with rest and can be exacerbated by physical or mental activity (CDC, 2023). ME/CFS is recognized as having a spectrum, with a gradient of clinical phenotypes and varying etiologies. The treatment of ME/CFS is hindered by the absence of a known specific cause and pathophysiology, and by the lack of diagnostic biomarkers for ME/CFS disease subsets. However, there is growing evidence that immune system abnormalities are found in a substantial number of affected individuals and may contribute to disease pathophysiology (Hornig et al., 2015; Mandarano et al., 2020; Montoya et al., 2017; Nijs et al., 2014). Additionally, gastrointestinal (GI) symptoms of unknown etiology have been found to be common in ME/CFS (Whitehead et al., 2002; Komaroff and Buchwald, 1991; Aaron

Abbreviations: ME/CFS, myalgic encephalomyelitis/chronic fatigue syndrome; LPS, lipopolysaccharide; sCD14, soluble CD14; LBP, LPS-binding protein; FABP2, intestinal fatty acid-binding protein; ELISA, enzyme-linked immunosorbent assay; TLR, toll-like receptor; EndoCAb, endotoxin-core antibody.

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et al., 2000, 2001). A limited number of studies on ME/CFS patients provide evidence for changes in gut bacterial population (Xiong et al., 2023; Guo et al., 2023; Shukla et al., 2015; Giloteaux et al., 2016; Fremont et al., 2013; Nagy-Szakal et al., 2017), elevated circulating microbial DNA (Shukla et al., 2015), and increased immune responses to bacterial lipopolysaccharide (LPS) (Maes et al., 2007; Vogl et al., 2022), suggesting gut microbial dysbiosis and possible alterations in mucosal barrier function. However, the contributing factors and pathogenic relevance of these findings remain unclear.

Gut epithelial surfaces are in constant contact with an abundance of potentially immunogenic microbial and dietary products. Compromised intestinal barrier integrity can therefore result in systemic innate and adaptive immune responses that are a consequence of microbial translocation from the gut lumen into circulation (Estes et al., 2010). Systemic immune activation in response to microbial translocation is a noted component of HIV infection and inflammatory bowel disease (Brenchley and Douek, 2012). In the present study, we aimed to investigate 1) whether ME/CFS is associated with specific systemic adaptive and innate immune responses that may arise due to compromised intestinal barrier function and microbial translocation across the gut epithelial barrier, 2) whether ME/CFS patients exhibit increased gut epithelial cell damage that may contribute to microbial translocation, 3) whether and how the observed immune responses are affected by maximal exercise, which is known to increase intestinal permeability and microbial translocation, and 4) whether metabolic differences in response to exercise may highlight molecular pathways to provide further insights into the observed immune responses in the context of compromised gut epithelium and microbial translocation. Utilizing data from two independent pairs of patient and control cohorts, we identify a state of downregulated acute-phase innate immune response and upregulated humoral immune response to intestinal damage-associated microbial translocation in ME/CFS that may be mediated by IL-10 immunoregulatory activity and specific immune-relevant defects in metabolic pathways.

2. Methods

2.1. Study participants

The study's primary cohorts included 131 individuals with ME/CFS and 86 healthy control participants. These individuals were originally recruited and samples collected under institutional review board-approved protocols as part of a collaboration between Solve ME/CFS Initiative, a group of CFS clinical sites, and GlaxoSmithKline, as previously described in detail (Irlbeck et al., 2014). Individuals with ME/CFS met both the Fukuda and the Canadian diagnostic criteria (Fukuda et al., 1994; Carruthers, 2007). In addition, ME/CFS participants reported having an initial presentation of flu-like illness or an acute (48 h) or subacute (4 weeks) onset, fatigue that persisted for at least six months, post-exertional malaise lasting >24 h, and significant cognitive impairment in short-term memory and concentration. Furthermore, the ME/CFS participants were required to meet 2 of the 3 following benchmarks on the RAND-36 quality of life survey: vitality <35, social functioning <62.5, role-physical <50. The healthy controls were from the same neighborhood or resided within the same zip code or neighboring zip code as the individuals with ME/CFS, but did not reside in the same household, at the time of recruitment. Subjects were excluded from the study if they had a body mass index (BMI) >40 kg/m^2, cancer, a history of substance or alcohol abuse <2 years before onset of ME/CFS, untreated hypothyroidism, or a major psychiatric disorder. Individuals who had a history of liver disease, liver function blood test results (AST (aspartate transaminase), ALT (alanine transaminase), ALP (alkaline phosphatase), total protein, albumin, globulin, and bilirubin) outside the normal range, or a recent infection were excluded from the study. Subjects with a chronic inflammatory disease, such as systemic lupus erythematosus, rheumatoid arthritis, or inflammatory bowel disease were excluded. Female subjects who were pregnant, <3 months postpartum, or currently lactating were not included in the study.

In addition, the study included a separate and independent second set of cohorts, comprising of 9 individuals with ME/CFS and 7 healthy control subjects who participated in a maximal exercise challenge. Plasma samples were available from these participants prior to and following the exercise routine. The samples were collected with written informed consent under institutional review board-approved protocols at the Marshfield Clinic and University of Wisconsin-Madison. Individuals with ME/CFS met the Fukuda case definition criteria (Fukuda et al., 1994) and standard medical history was reviewed and a physical exam was conducted for each study participant to rule out any major illnesses other than ME/CFS. Routine blood and urine chemistry tests were used to screen for exclusionary medical conditions or other conditions that may explain the patients' symptoms. Exclusionary conditions included untreated hypothyroidism, sleep disorders, side effects of medication, potential relapsing of past medical issues (e.g., Lyme disease, hepatitis B or C, etc.), major psychiatric issues, including major depressive disorder with psychotic or melancholic features, alcohol or other substance abuse within two years of the onset of ME/CFS, and severe obesity as defined by BMI>40 kg/m^2. In addition, potential participants were excluded if they were currently using immunomodulatory medications, stool softeners, laxatives, anti-diarrheal agents, antibiotics, probiotics, or opioids, had a history of cardiovascular disease or uncontrolled hypertension, or were currently experiencing fatigue sufficient to interfere with or preclude exercise testing. Patients were asked to confirm the absence of exclusionary medications on the day of testing and to list other current medication use. The control group was comprised of healthy people who were evaluated for general health to confirm that that they did not meet ME/CFS case definition criteria. Participants performed a maximal exercise test on an electronically braked cycle ergometer (Sensormedics, Loma Linda, CA), following the previously described protocol (Shukla et al., 2015; Cook et al., 2012). Blood plasma from participants was acquired immediately before the start of the exercise challenge and at 15 min post peak effort.

All biospecimens in this study were kept at −80 °C until use to maintain stability. This study was approved by the Institutional Review Board of Columbia University Medical Center.

2.2. Assays

Plasma levels of IgG, IgA, and IgM anti-endotoxin (LPS)-core antibodies (EndoCAb) (Hycult Biotech, Uden, The Netherlands), lipopolysaccharide (LPS)-binding protein (LBP) (Hycult Biotech), soluble CD14 (sCD14) (R&D Systems, Minneapolis, MN), and intestinal fatty acid-binding protein (FABP2) (R&D Systems) were determined by ELISA according to the manufacturers' protocols, as we have previously described (Uhde et al., 2016).

Levels of IgG, IgA, and IgM antibodies to the gliadin protein fraction of dietary wheat were measured separately by the enzyme-linked immunosorbent assay (ELISA) as we have previously described (Uhde et al., 2016; Huebener et al., 2015). IgG, IgA, and IgM antibodies to casein were measured separately using a similar protocol to that for antibodies to gliadin, with the difference that the plates were coated with a 10 μg/mL solution of bovine milk casein (Sigma-Aldrich, St. Louis, MO).

IgG, IgA, and IgM antibodies to bacterial flagellin were also measured separately using the protocol for detecting antibodies to gliadin, with the following modification: plates were coated with a 2 μg/mL solution of highly purified flagellin from *Salmonella typhimurium* (InvivoGen, San Diego, CA).

Concentrations of IL-10, IFN-γ, and TNF-α cytokines were measured by a high sensitivity ELISA according to the manufacturers' protocol (Quansys Biosciences, Logan, UT). LPS concentration was measured using the Kinetic-QCL kinetic chromogenic limulus amebocyte lysate (LAL) assay (Lonza, Basel, Switzerland).

2.3. Metabolomic profiling

Global metabolomics for plasma samples from each participant before and after the exercise challenge was completed using the Metabolon Platform (Metabolon, Morrisville, NC), as previously described (Ford et al., 2020). Results for the profiling of 897 metabolites were organized into 8 super pathways and 80 sub-pathways.

2.4. Data analysis

Data were tested for normality of distribution, equality of variances between groups, and outliers prior to comparative analyses. Differences between groups for the demographic data were analyzed by the two-tailed Fisher's exact test (sex) or the *t*-test (age and BMI). Group differences for the assay data were assessed by the analysis of covariance (ANCOVA), using the general linear model to account for the potential confounding effect of differences in age, sex, and BMI. Data were log transformed as required prior to analysis to meet statistical assumptions of ANCOVA, including equality of variance and normality. Partial correlation analysis was performed using linear regression and Spearman's *r*, controlling for the covariates. The effect of the exercise challenge was assessed by the Wilcoxon matched-pairs test. All *P* values were 2-sided, and differences were considered statistically significant at $P < 0.05$. Statistical analyses were performed with Prism 9 (GraphPad, San Diego, CA) and Minitab 17 (Minitab, State College, PA) software.

Statistical analysis of metabolomic data was performed in OmicSoft Array Studio (Qiagen, Hilden, Germany). An estimate of the false discovery rate (*q*-value) was calculated to account for multiple comparisons and significance was defined as $P < 0.05$ and $q < 0.10$. A repeated measures random forest analysis was performed to compare the pre- and post-exercise groups. A multivariate principal component analysis (PCA) was done on the dataset to reduce data dimensionality and to assess clustering.

3. Results

3.1. Study participants

The demographic characteristics of the study cohorts are included in Table 1. The ME/CFS and healthy control cohorts were not significantly different in terms of age, sex, race, or body mass index.

3.2. Elevated antibody response to microbial antigens in ME/CFS

When compared with the healthy control cohort, the ME/CFS group had significantly higher levels of EndoCAb (anti-LPS) IgG, IgA, and IgM ($P < 0.0001$, $P < 0.0001$, and $P = 0.008$, respectively) (Fig. 1a–c). Furthermore, the levels of IgG, IgA, and IgM antibodies to flagellin were significantly elevated in the ME/CFS cohort in comparison with the healthy control group ($P = 0.001$, $P = 0.004$, and $P = 0.006$, respectively) (Fig. 1d–f). The increased antibody responses to flagellin correlated with the elevated EndoCAb in the ME/CFS cohort for each isotype ($r = 0.280$, $P = 0.001$ for IgG; $r = 0.303$, $P = 0.0005$ for IgA; $r = 0.487$, $P < 0.0001$ for IgM).

3.3. Enhanced antibody response to dietary antigens in ME/CFS

To further ascertain the presence of enhanced systemic antibody reactivity to translocated antigens likely derived from the gastrointestinal tract, levels of antibody response to two of the most ubiquitous and commonly consumed dietary proteins, wheat gliadin and milk casein, were measured. In the ME/CFS cohort, IgG, IgA, and IgM antibodies to native gliadin were significantly higher than in the healthy control group ($P = 0.001$, $P = 0.002$, and $P = 0.016$, respectively) (Fig. 2a–c). Similarly, IgG, IgA, and IgM antibodies to casein were significantly elevated in comparison with healthy controls ($P = 0.013$, $P = 0.037$, and $P = 0.001$ respectively) (Fig. 2d–f). There were significant correlations between the same isotypes of anti-gliadin and anti-casein antibodies ($r = 0.208$, $P = 0.018$ for IgG; $r = 0.208$, $P = 0.018$ for IgA; $r = 0.372$, $P < 0.0001$ for IgM).

Additionally, there were significant correlations, but only for the IgM isotype, between the elevated antibody responses to the microbial and the dietary antigens. IgM EndoCAb and anti-flagellin antibodies correlated with the IgM antibodies to gliadin ($r = 0.542$, $P < 0.0001$, and $r = 0.471$, $P < 0.0001$, respectively) and to casein ($r = 0.327$, $P = 0.0002$, and $r = 0.355$, $P < 0.0001$, respectively).

3.4. Increased intestinal epithelial cell turnover

In comparison with the healthy control group, plasma concentrations of FABP2, a specific marker of intestinal epithelial cell damage and turnover rate (Pelsers et al., 2005), were significantly elevated in the ME/CFS cohort ($P = 0.006$) (Fig. 3).

3.5. Lack of LBP and sCD14 acute-phase activation in ME/CFS

In contrast with the humoral immune responses towards the LPS and flagellin microbial antigens, plasma levels of neither LBP nor sCD14 acute-phase responses were significantly elevated in individuals with ME/CFS in comparison with healthy controls (Fig. 4a and b).

3.6. Enhanced antibody and suppressed LBP and sCD14 responses to microbial translocation in high-intensity exercise

Levels of the above-examined markers of innate and adaptive immune activation were also assessed in the secondary ME/CFS and healthy control groups in response to high-intensity exercise, which is known to enhance intestinal permeability and microbial translocation. The healthy control group displayed an expected significant increase in LBP ($P = 0.007$) and sCD14 ($P = 0.008$) levels following exercise (Fig. 5a). However, there was a lack of significant increase in LBP levels in the ME/CFS group after exercise and a comparatively more modest rise in sCD14 in ME/CFS ($P = 0.021$) in comparison with healthy controls (Fig. 5b), mirroring the findings from the cohorts at rest. In line with these results, individuals with ME/CFS, but not healthy controls, exhibited elevated circulating levels of LPS ($P = 0.008$) (Fig. 5c). In contrast with the dampened LBP and sCD14 responses in the ME/CFS group, there was a significantly increased IgM antibody response to microbial and dietary antigens in ME/CFS patients after exercise ($P = 0.004$ for LPS; $P = 0.004$ for gliadin) (Fig. 5d and e).

Table 1
Demographic and clinical characteristics of study cohorts.

Subject group	Number of subjects	Age—years [SD]	Female sex—no. (%)	White race—no. (%)	BMI—(kg/m^2) [SD]	Duration of illness—years [SD]
Primary cohorts						
ME/CFS	131	50.0 [11.4]	89 (68)	117 (91)	26.0 [5.5]	12.7 [9.6]
Healthy	86	50.0 [12.8]	68 (79)	80 (93)	26.5 [6.8]	–
Exercise challenge cohorts						
ME/CFS	9	44.0 [15.5]	8 (89)	9 (100)	21.6 [2.7]	12.8 [7.5]
Healthy	7	41.4 [17.8]	4 (57)	7 (100)	24.2 [3.4]	–

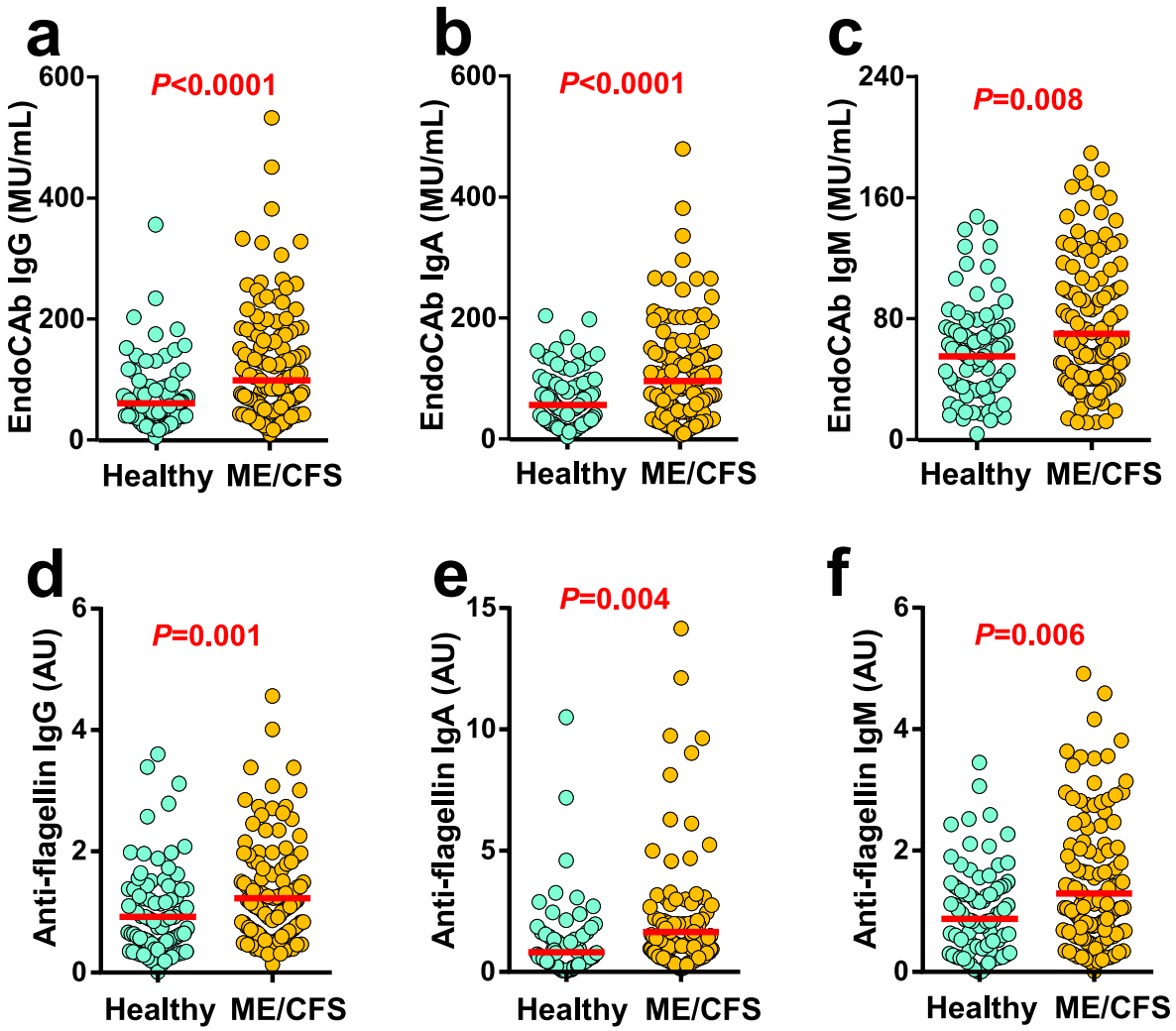

Fig. 1. (a–f) Humoral immune response to microbial antigens. Plasma levels of (a) IgG antibody to LPS (EndoCAb IgG), (b) IgA antibody to LPS (EndoCAb IgA), (c) IgM antibody to LPS (EndoCAb IgM), (d) IgG antibody to flagellin, (e) IgA antibody to flagellin, and (f) IgM antibody to flagellin in cohorts of healthy controls (n = 86) and ME/CFS (n = 131) patients. Data are shown as scatterplots with the red bar indicating the median for each cohort. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

In light of these results, we examined the concentrations of the immunoregulatory cytokine, IL-10, which is known for playing a role in downregulating the expression of Th1 cytokines and further inflammatory responses, but also having B cell and antibody response enhancement potential (Saraiva and O'Garra, 2010; Heine et al., 2014). Levels of IL-10 increased significantly in response to exercise only in the ME/CFS group ($P = 0.011$) (Fig. 5f). A significant change in the concentrations of IFN-γ or TNF-α cytokines in response to exertion was not observed in either group (data not shown).

3.7. Metabolomic changes consistent with physical exertion

In comparison to baseline, levels of 364 (40.6%) metabolites exhibited a significant fold change after exercise in the healthy control group, while this number was 265 (29.5%) in the ME/CFS cohort. Levels of 194 metabolites (21.6%) had a significant fold change after exercise in both the ME/CFS and control cohorts. When a repeated measures random forest analysis was performed to compare the pre- and post-exercise control groups (with predictive accuracy of 92.86%), among the top 30 biochemicals contributing to group separation were those involved in the metabolism of carbohydrates, lipids, nucleotides, amino acids, and energy. Notably, several closely related organic acids including lactate, pyruvate, succinate, and malate were among the top-ranked biochemicals, pointing to the important role of energy metabolism in differentiating the pre- and post-exercise groups (Fig. 6a). Similar to healthy controls, the top-ranked biochemicals contributing to separation of the pre- and post-exercise ME/CFS groups (with predictive accuracy of 88.9%) were primarily involved in the metabolism of carbohydrates, lipids, nucleotides, amino acids, and energy (Fig. 6b).

Principal component analysis (PCA) of the complete metabolomic data pointed to a similar leftward shift for the post-exercise healthy control and ME/CFS study participants relative to their matched pre-exercise state (Fig. 6c). In addition, the data pointed to some degree of metabolic stratification between the ME/CFS and control groups.

3.8. Deficient glucose and citrate metabolic responses

Levels of 51 metabolites (5.7% of total) exhibited a significant fold change after exercise only in the ME/CFS group ($P < 0.05$), whereas 131 metabolites (14.6% of total) were altered after exercise only in the control group ($P < 0.05$). Of specific relevance to our earlier data were the levels of glucose and citrate, which are of particular importance to the proper functioning of innate immune responses. During high-intensity exercise, enzymatic breakdown of glycogen into glucose provides acetyl-CoA molecules that enter the Krebs cycle to generate citrate and, subsequently, energy in the form of ATP. Plasma glucose levels

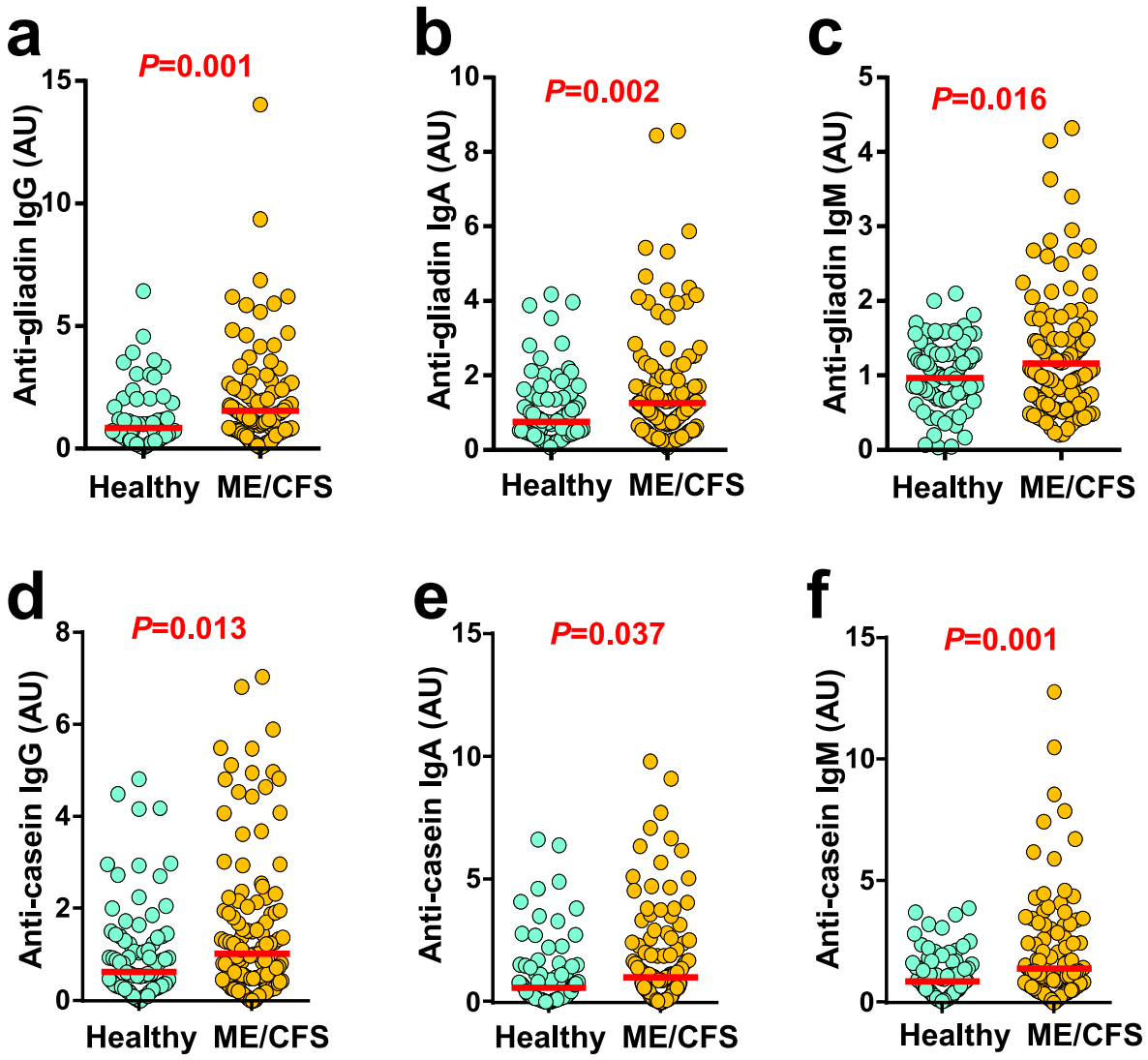

Fig. 2. (a–f) Humoral immune reactivity to dietary antigens. Plasma levels of (a) IgG antibody to wheat gliadin, (b) IgA antibody to gliadin, (c) IgM antibody to gliadin, (d) IgG antibody to milk casein, (e) IgA antibody to casein, and (f) IgM antibody to casein in healthy controls (n = 86) and individuals with ME/CFS (n = 131). Data are shown as scatterplots with the red bar indicating the median for each cohort. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

were significantly elevated in the healthy control group in response to exercise ($P = 0.006$), but not in the ME/CFS group (Fig. 6d). Similarly, citrate levels were significantly elevated only in the healthy control group after exercise ($P = 0.0004$) (Fig. 6e). The citrate response to exercise correlated negatively with IL-10 ($r = -0.684$, $P = 0.004$) and positively with LBP ($r = 0.551$, $P = 0.029$) responses.

4. Discussion

The observed increase in antibody responses to both microbial and dietary antigens along with higher expression of FABP2, reflecting greater epithelial cell damage and turnover rate, point to enhanced translocation of gut luminal antigens across a compromised intestinal barrier in ME/CFS. In light of these findings, we expected an increase in the activation of LBP and sCD14 acute-phase immune responses as further indicators of the translocation of immunogenic microbial products across the epithelial barrier. Translocated circulating LPS can result in the rapid secretion of LBP by gastrointestinal and hepatic epithelial cells, as well as sCD14 by $CD14^{+}$ monocytes/macrophages (Brenchley and Douek, 2012). LBP and sCD14 also bind to and are released in response to other microbial constituents, serving as general markers of microbial translocation (Schumann, 2011). Despite the elevation in the antibody responses, however, no significant differences in levels of LBP or sCD14 were seen in comparison with controls. A prior study by us on the same cohorts had similarly found a lack of significant difference between ME/CFS patients and controls in the levels of C-reactive protein (CRP), another highly sensitive acute-phase immune response protein that binds a variety of microbial ligands (Uhde et al., 2018). We speculated that these observations may be driven either by a lack of significant microbial translocation at the time of blood draw (since antibody reactivities reveal comparatively little information regarding acute exposure), or a by a defect in specific acute-phase immune responses in ME/CFS.

To explore these possibilities, we examined ME/CFS patients and healthy controls in a maximal exercise challenge. Endurance exercise in healthy adults is known to disrupt the gut mucosal barrier, which is associated with increased intestinal permeability, translocation of microbial molecules into central circulation, and endotoxemia, resulting in systemic immune activation (Camus et al., 1997; Jeukendrup et al., 2000; Yeh et al., 2013; Phua et al., 2015). Therefore, exercise challenge is an especially useful tool for characterizing the immune response to enhanced gut permeability and microbial translocation. As expected, the

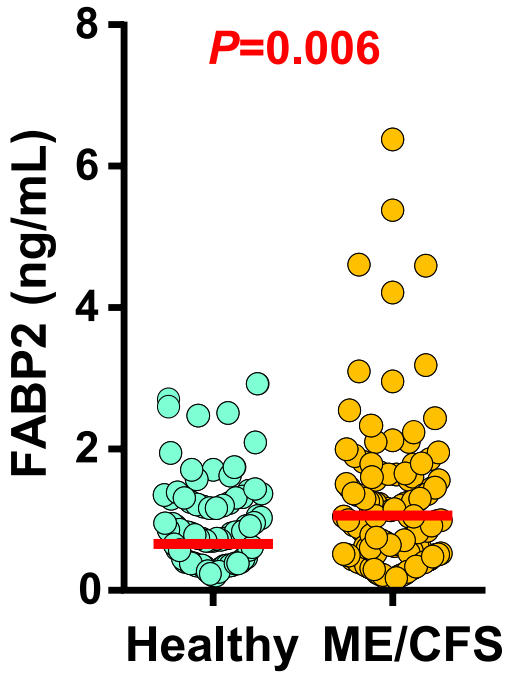

Fig. 3. Intestinal cell damage. Plasma levels of FABP2, a specific marker of gut epithelial cell damage and turnover rate, in cohorts of healthy controls (n = 86) and ME/CFS (n = 131) patients. Data are shown as scatterplots with the red bar indicating the median for each cohort. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

maximal exercise challenge resulted in a significant increase in LBP and sCD14 acute-phase responses in the control group of participants. However, a similar increase was lacking in ME/CFS patients, strengthening the evidence for a downregulated acute-phase response in ME/CFS. The observed concomitant rise in circulating LPS levels after exercise in the ME/CFS cohort, but not healthy controls, provided further evidence for the insufficiency of acute-phase innate immune responses in ME/CFS at neutralizing circulating microbial products.

These data could be highly relevant to the clinical history and presentation of ME/CFS. LBP and sCD14 play a key role in the innate immune response to bacterial challenge by binding to TLR4 and instigating a rapid inflammatory response, as well as neutralizing LPS by transferring it to lipoproteins to inhibit its stimulatory effects and downregulate host defense under other conditions (Wurfel et al., 1994, 1995). In mice, LBP is essential for controlling the multiplication of bacteria and for survival during *Salmonella* infection (Jack et al., 1997; Heinrich et al., 2001; Fierer et al., 2002). Similarly, CD14 disruption has been shown to aggravate disease and shorten survival in response to infection in mice (Echchannaoui et al., 2005). In fact, "susceptibility to infections" happens to be among the most common complaints from ME/CFS patients (Institute of Medicine of the National Academies, 2015). The data may also be highly relevant to the symptomatic response to exertion in ME/CFS, as certain symptoms of endotoxemia such as fatigue, cognitive changes, headache, nausea, increases in heart rate, and decreases in blood pressure mirror those reported by people with ME/CFS during post-exertional malaise (Hartle et al., 2021). Furthermore, LPS has been shown to bind directly to TLR4 on the luminal surface of brain blood vessels, resulting in local cytokine secretion in the brain, activating the microglia to displace inhibitory synapses (Chen et al., 2012). In HIV infection, the observed microbial translocation, which is linked to a compromised intestinal epithelium, is associated with increased systemic immune activation, neuroinflammation, and cognitive deficits (Sandler and Douek, 2012; Vera et al., 2016). It is conceivable that such a pathway could contribute to some of the neurocognitive symptoms in

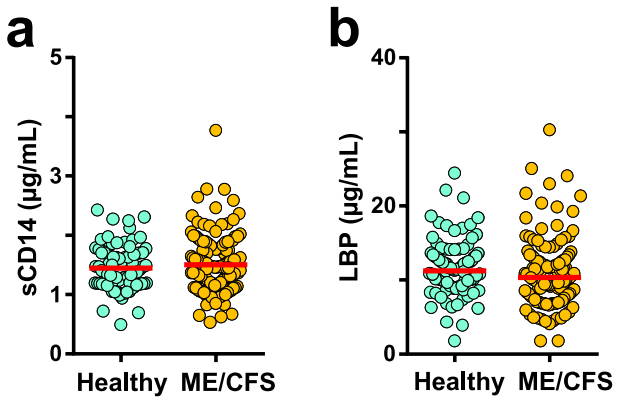

Fig. 4. Markers of systemic acute-phase immune response to microbial constituents. Plasma levels of (a) sCD14, and (b) LBP in cohorts of healthy controls (n = 86) and ME/CFS (n = 131) patients. Data are shown as scatterplots with the red bar indicating the median for each cohort. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

ME/CFS, as brain imaging studies confirm low-level neuroinflammation and altered brain function in conjunction with impaired cognitive performance and other associated symptoms in affected individuals, including in response to exercise (Nakatomi et al., 2014; Mueller et al., 2020; Aoun Sebaiti et al., 2022; Cook et al., 2017).

Memory B cells in peripheral blood can contribute to the expression of IgM antibodies that target a diverse array of antigens, offering an early line of defense against potential pathogens (Lanzavecchia et al., 2006). For example, exposure to hypomethylated CpG sequences, which are abundant in bacterial and viral genomes, can result in TLR9-dependent proliferation and differentiation of these B cells, independent of direct interaction with their respective antigens or T cell involvement (Bernasconi et al., 2002; Capolunghi et al., 2013). Acute microbial translocation from the gut would be expected to enhance the secretion of IgM antibodies in the periphery via this pathway (Capolunghi et al., 2013; Bailey et al., 2004). Following the exercise routine, we observed an increased IgM antibody response to microbial and dietary antigens in the ME/CFS cohort, in contrast to a more muted response in the healthy control group. The observed enhancement of the innate-like IgM antibody reactivity to dietary and microbial antigens in ME/CFS may be a mechanism directed at compensating for the lack of adequate LBP and sCD14 responses in order to counteract the increased circulating microbial concentrations.

To explore potential mechanisms associated with the dampened LBP and sCD14 acute-phase responses and the enhanced humoral immune response in the context of ME/CFS, we proceeded to examine IL-10 immunoregulatory cytokine and immune-relevant metabolic changes in response to exercise. IL-10 is a primarily anti-inflammatory cytokine involved in immunoregulation. It is known to be expressed in response to microbial products through different signalling pathways and to be particularly important as a key mediator of intestinal immune homeostasis (Saraiva and O'Garra, 2010; Engelhardt and Grimbacher, 2014; Paul et al., 2012). At least three previous studies have reported on enhanced IL-10 expression in the context of ME/CFS, including in response to exercise (Light et al., 2009; Visser et al., 2001; Nakamura et al., 2010). IL-10 has been shown to inhibit the activation of human monocytes and to limit inflammation in murine disease models (Iwata et al., 2011; Fillatreau et al., 2002; Mauri et al., 2003). Specifically, it blocks the induction of NF-κB activity and production of pro-inflammatory cytokines by LPS and other bacterial products, thereby limiting the immune response to pathogens and having the capacity to prevent damage to host (Saraiva and O'Garra, 2010). IL-10 also promotes the T cell independent production of IgM and IgG by plasmablasts when induced via activation through TLR9 by microbial DNA

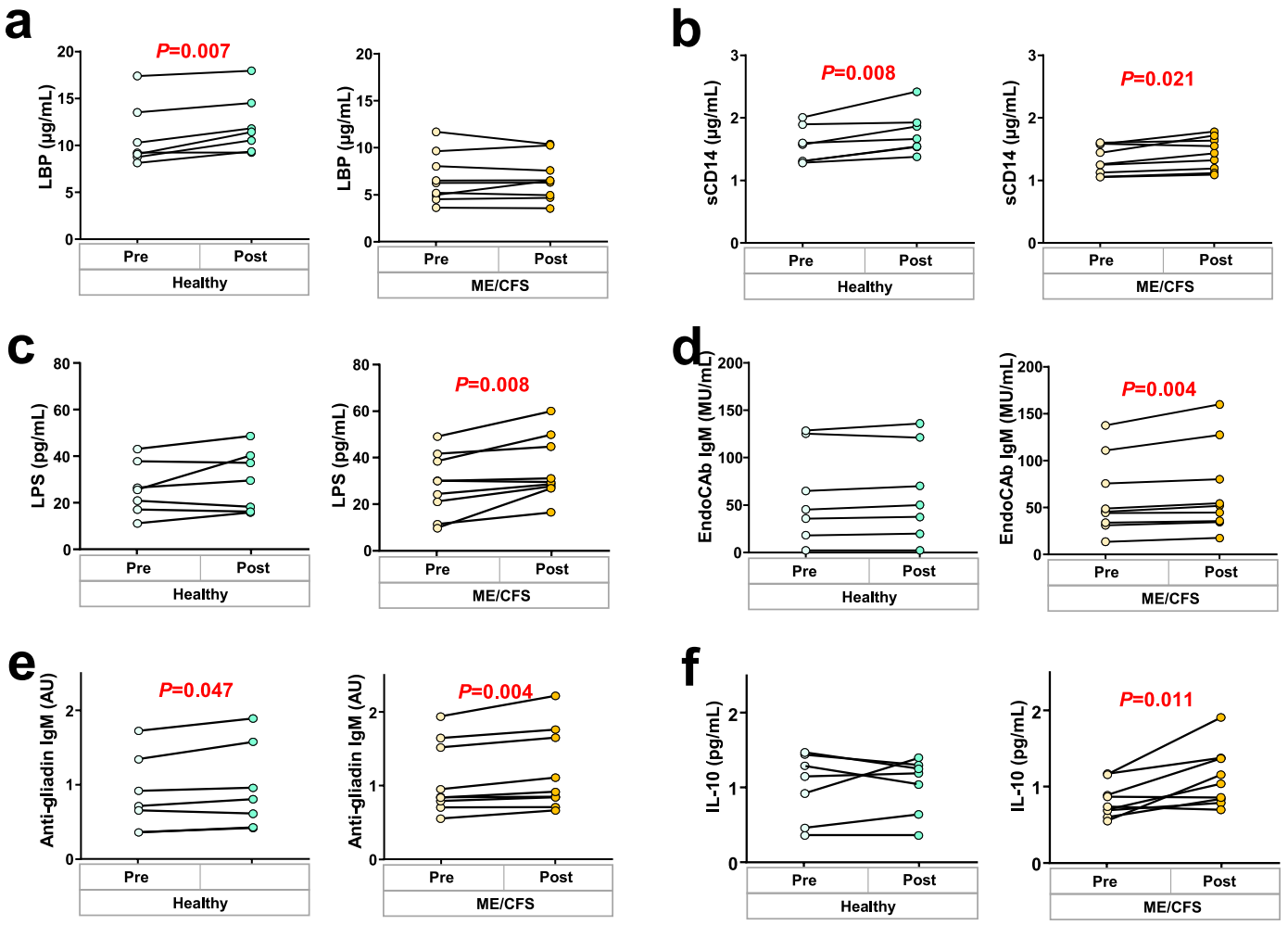

Fig. 5. Markers of immune activation and microbial translocation in response to the exercise challenge. (a–f) Levels of LBP (a), sCD14 (b), LPS (c), IgM antibody to LPS (EndoCAb IgM) (d), IgM antibody to gliadin (e) and IL-10 (f) before and after the maximal exercise challenge in unaffected controls (n = 7) and individuals with ME/CFS (n = 9). Each individual is represented by a filled circle and the two points corresponding to the same individual are connected by a line.

(Heine et al., 2014; Vasquez et al., 2015; He et al., 2004). As such, the observed increase in IL-10 levels in ME/CFS during maximal exercise may be part of a mechanism to limit the inflammatory reaction triggered by circulating microbial products (such as those mediated by LBP and sCD14), while enhancing the antibody-mediated clearance of translocated microbial and dietary antigens, as indicated by our study.

The ability of immune cells to respond to environmental events, such as increased circulating microbial products like LPS, is closely linked to changes in cellular metabolism (Buck et al., 2017). In this context, the significant increase in plasma glucose among control subjects of this study is not only reflective of the required demand for energy during exercise, but as several studies have shown, it is also in line with the enhanced glucose uptake needed for optimal innate immune activation, including that of pro-inflammatory macrophages and dendritic cells in response to microbial products (Grosick et al., 2018; Fukuzumi et al., 1996; Jantsch et al., 2008; Stunault et al., 2018). Heightened glucose conditions appear to activate monocytes, contributing to the expression of an M1 macrophage pro-inflammatory phenotype needed during breach of the intestinal barrier and enhanced microbial translocation (Grosick et al., 2018). In addition, previous work has shown that glucose deprivation can upregulate surface PD-1, which induces IL-10 production upon PD-L1 binding (Chang et al., 2013; Wherry and Kurachi, 2015; Said et al., 2010). IL-10, in turn, blocks aerobic glycolysis in dendritic cells and macrophages, further abrogating their acute-phase anti-bacterial response (Krawczyk et al., 2010; Ip et al., 2017). As such, the observed lack of an optimal glucose response in the ME/CFS cohort may be directly related to the upregulation of IL-10 and the suppressed acute-phase innate immune responses in our study.

Another metabolite of crucial importance to innate immune function is the Krebs cycle intermediate citrate. Citrate is produced in the Krebs cycle by condensation of oxaloacetate and acetyl-CoA, the latter being generated from glycolytic pyruvate or from fatty acids. Most of the work on the immunomodulatory role of citrate has been done in the context of the effect of LPS on macrophages, assigning a key role to it in sustaining macrophage inflammatory responses and contribution to M1 polarization (Viola et al., 2019; Tannahill et al., 2013; Infantino et al., 2014). Accordingly, the observed lack of citrate elevation in response to exercise in the ME/CFS cohort can be expected to have further inhibitory effects on innate immune activation of macrophages and other cells, in line with the observed downregulation of acute-phase anti-microbial response and IL-10 upregulation in this study.

It is important to emphasize that because ME/CFS is a highly heterogeneous spectrum disorder, these findings are likely relevant to a subset(s) of affected individuals rather than the entire patient population (Missailidis et al., 2019). Furthermore, it should be acknowledged that for the exercise challenge arm of the study, the relatively small sample size and the use of only the Fukuda criteria for recruitment, are noteworthy limitations. However, the inclusion of an exercise interventional component relevant to a hallmark of ME/CFS, namely post-exertional malaise, is a significant strength that provides further

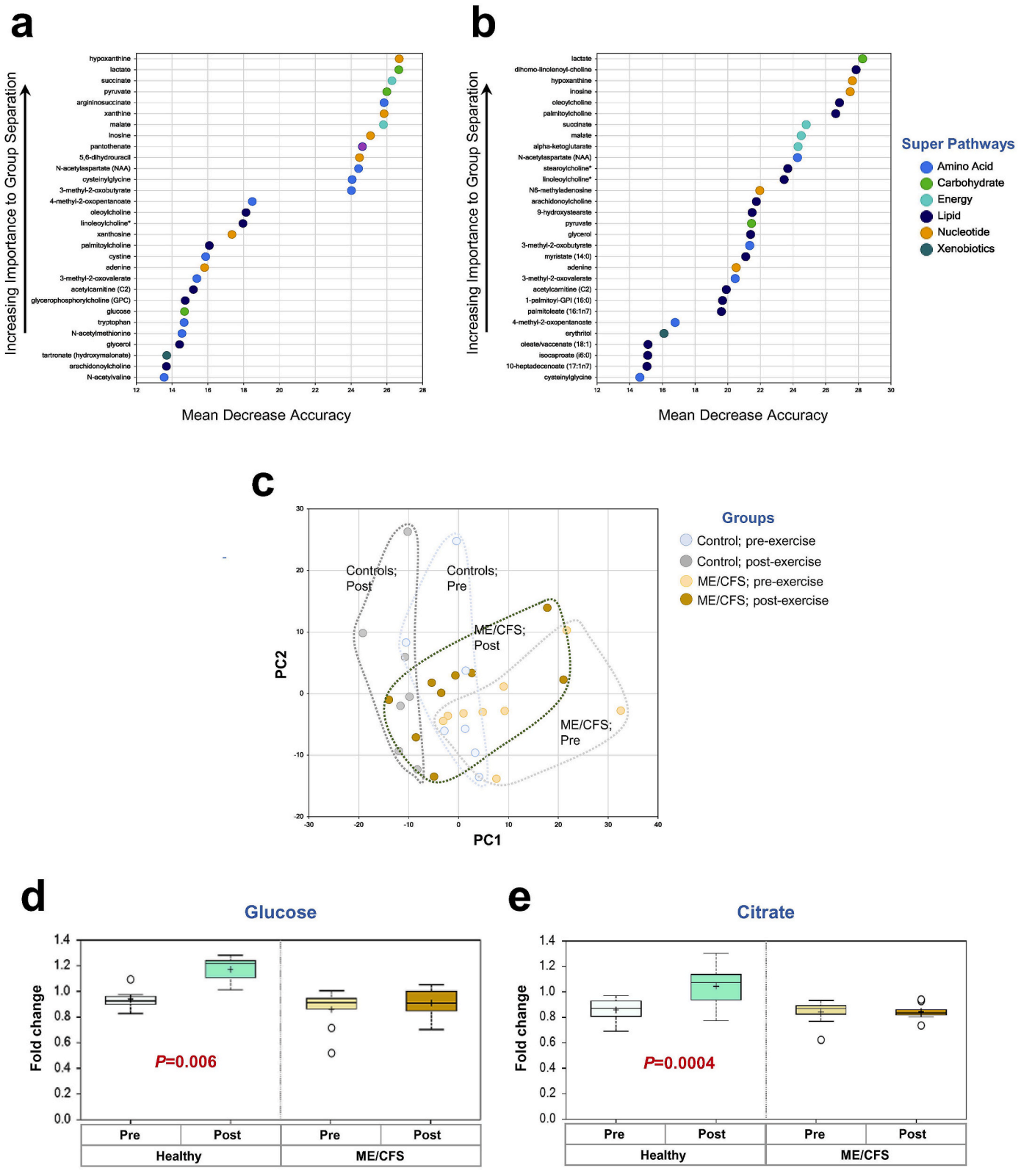

Fig. 6. Metabolomic response to exercise challenge. (a–b) Biochemical importance plots using repeated measures random forest analysis to compare the pre- and post-exercise healthy control participants (n = 7) (a) and the pre- and post-exercise ME/CFS participants (n = 9) (b), showing the top 30 biochemicals contributing to the separation of pre- and post-exercise groups for each study cohort. (c) Principal component analysis (PCA) of the complete metabolomic data, showing the degree of metabolic stratification and the shift for the post-exercise study participants relative to their pre-exercise state for healthy controls and individuals with ME/CFS. (d–e) Box-and-whisker plots showing the plasma concentration fold change for glucose (d) and citrate (e) in response to exercise for the control and ME/CFS cohorts.

validation of our data, serving as a potential model for designing future studies. Nevertheless, there is clearly a need for studies with larger and even better characterized cohorts to gain a clearer picture of the level of applicability of these data to the ME/CFS population, especially in the context of response to exertion. As post-exertional malaise in ME/CFS can sometimes be delayed for 24–72 h and last for extended periods, it is also imperative that future studies include further longitudinal analyes of immunologic and metabolic responses.

In summary, our data provide evidence for a state of suppressed immune and metabolic responses to intestinal cell damage and microbial translocation across the gut epithelial barrier in ME/CFS. The identified dysfunctional responses may carry particular relevance in the context of ME/CFS clinical presentation, such as susceptibility to infections, flu-like symptoms, gastrointestinal dysfunction, and cognitive deficits. In moving forward, we envision an opportunity to build on these data by further investigating their relevance to ME/CFS symptom profile and clinical history, exploring the mechanisms responsible for increased susceptibility to mucosal barrier dysfunction and associated immunoregulatory and metabolic responses, assessing the value of the identified biomarkers for monitoring the response to specific treatment strategies, and examining the potential therapeutic utility of targeting the identified defects in gut barrier function, anti-microbial immunologic reaction, and metabolic response to exertion in affected individuals.

Author contributions

Study concept and design: AA, SDV; *Contribution to study design:* AI, MU, PHG, DBK, SKS, SDV; *Acquisition of data:* AI, MU, SDV, AA; *Analysis and interpretation of data:* AI, MU, SKS, SDV, AA; *Drafting of the manuscript:* MU, SDV, AA; *Critical revision of the manuscript for important intellectual content:* AI, MU, PHG, SKS, SDV, AA; *Statistical analysis:* AI, MU, AA; *Administrative, technical, or material* support*:* PHG, DBK, SKS, SDV, AA; *Obtained* funding*:* AA, SKS*; Study supervision:* AA.

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Declaration of competing interest

AA reports participation on advisory boards for the National Institutes of Health, Global Lyme Alliance, Roche, Everlywell, and Veravas.

Data availability

Data will be made available on request.

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