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Sex-specific antioxidant biomarker depletion in patients with a history of mild traumatic brain injury

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Abstract

Individuals with a history of mild traumatic brain injury (mTBI) are at an increased risk for neurodegenerative disease, suggesting that intrinsic neuroprotective mechanisms, such as the endogenous antioxidant reservoir, may be depleted long-term after mTBI. Here, we retrospectively analyzed symptoms and blood antioxidants in patients with a history of mTBI who presented to Resilience Code, a sports medicine clinic in Colorado. Significant decreases in alpha-tocopherol, selenium, linoleic acid, taurine, docosahexaenoic acid, and total omega-3 were measured in the total mTBI population versus controls. Male mTBI patients showed depletion of a larger array of antioxidants than females. Patients with a history of mTBI also reported significantly worsened emotional, energy, head, and cognitive symptoms, with males displaying more extensive symptomology. Multiple or chronic mTBI patients had worsened symptoms than single or acute/subchronic mTBI patients, respectively. Finally, male mTBI patients with the largest reductions in polyunsaturated fatty acids (PUFAs) displayed worse symptomology than male mTBI patients with less depletion of this antioxidant reservoir. These results demonstrate that antioxidant depletion persists in patients with a history of mTBI and these deficits are sex-specific and associated with worsened symptomology. Furthermore, supplementation with specific antioxidants, like PUFAs, may diminish symptom severity in patients suffering from chronic effects of mTBI.

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Lilia A. Koza: Writing – original draft, Methodology, Investigation, Formal analysis. **Allison N. Grossberg:** Writing – review & editing, Resources, Data curation, Formal analysis, Methodology. **McKensey Bishop:** Investigation, Data curation. **Chad Prusmack:** Writing – review & editing, Resources, Conceptualization. **Daniel A. Linseman:** Writing – review & editing, Resources, Conceptualization.

Declaration of competing interest

None

Supplementary materials

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Keywords

Traumatic brain injury; Antioxidants; Biomarkers; Symptomology; Polyunsaturated fatty acids

Introduction

Mild traumatic brain injury (i.e., concussion; mTBI) is the most commonly occurring severity of TBI. Of the 69 million TBIs that occur each year worldwide, approximately 80 % are mTBIs [1]. Primary injury, such as disruption of cellular integrity, calcium influx, edema, and diffuse axonal injury, that occur immediately following the impact, may not be severe with mTBI [2]. However, secondary injury pathology such as neuroinflammation, oxidative stress, blood-brain barrier (BBB) disruption, and excitotoxicity can lead to neuronal cell death following mTBI and may persist for weeks to months following an injury [3–6].

There is an increased risk of sustaining a future mTBI in high-risk individuals, such as athletes and military personnel, who already have a history of mTBI [7]. Subsequent mTBIs, termed repetitive mTBI (rmTBI), can exacerbate and prolong secondary injury [8,9]. Behavioral and cognitive symptoms have been reported in human subjects following mTBI and rmTBI; however, the duration of symptoms vary depending on the individual [10–12]. Furthermore, although symptoms may subside shortly following injury allowing an individual to return to their normal routine, underlying secondary injury pathology can persist.

Long-term secondary injury pathology from a single mTBI, and especially from rmTBI, has been identified as a risk factor for developing neurodegenerative disease later in life such as Alzheimer's disease (AD), Parkinson's disease, and chronic traumatic encephalopathy (CTE). Retired national football league athletes have a 3-fold increased risk of dying from either AD or amyotrophic lateral sclerosis (ALS) compared to non-athletes [13]. Cerebral atrophy, tau pathology, and proteino-pathies, which mirror pathology present in neurodegenerative disease, have also been observed in retired athletes with a history of rmTBI [14–16]. However, these findings come with limitations. There are inadequate data and reporting concerning player injuries and TBI classification in terms of severity and frequency. Furthermore, many of these studies were unable to collect information on environmental or genetic risk factors for neurodegenerative disease [17]. More research needs to be done to fully understand the relationship between mTBI and the risk for developing neurodegenerative disease.

Neuroinflammation and oxidative stress are frequently observed following mTBI and especially in those with a history of rmTBI. Persistent neuroinflammation and excitotoxicity in response to mTBI are well studied and result in the overproduction of reactive oxygen species (ROS), reactive nitrogen species (RNS), and lipid peroxides [18,19]. This leads to mitochondrial dysfunction, DNA damage, and ultimately, neuronal cell death. In response, the endogenous antioxidant system becomes strained resulting in antioxidant depletion [20]. Many studies have explored using antioxidants, such as N-acetylcysteine or alpha-tocopherol, to treat TBI in animal models, as well as a few studies in clinical trials [21–25].

Persistent antioxidant depletion likely contributes to lasting symptoms observed in patients with a history of mTBI; however, most clinical studies focused on antioxidant treatments for mTBI do not typically measure or report symptom resolution.

A few novel studies (2020-present) have begun to explore antioxidant levels following TBI. In a rat model of TBI, coenzyme-q9, coenzyme-q10, and α-tocopherol were found to be significantly reduced in brain tissue one-week post-severe TBI, although no differences were observed following mTBI [26]. Another study measured total antioxidant capacity in serum in humans one-week post-severe TBI and reported it may be indicative of survival outcomes [27]. These data indicate that there are important changes to the endogenous antioxidant system following TBI. However, to date, no studies have reported sex-specific differences in antioxidant depletion in patients with a history of mTBI. We hypothesized that patients with a history of mTBI would display significant antioxidant depletion and worsened symptoms, with sex-specific differences, when compared to control subjects with no history of mTBI. Given previous research that females may have a higher intrinsic antioxidant capacity, we expected that male patients with a history of mTBI would have greater antioxidant depletion, and subsequent worsened symptomology, than female patients with a history of mTBI. We aimed to test this hypothesis and fill a gap in the literature by retrospectively analyzing antioxidant levels in blood from male and female patients with a history of mTBI that presented to Resilience Code, a sports medicine clinic in Denver, Colorado.

Antioxidant biomarkers were measured in serum, plasma, red blood cells (RBCs), white blood cells (WBCs), and whole blood from male and female patients with a history of mTBI and compared to subjects with no history of mTBI. We also analyzed self-reported emotional, energy, head, and cognitive symptoms in these patients, based on answers reported on the Medical Symptom Questionnaire (MSQ). Furthermore, we performed a qualitative analysis to see if either the number of mTBIs sustained or the time since the most recent mTBI influenced symptom severity and frequency. Lastly, we compared symptomology in patients with a history of mTBI displaying different magnitudes of polyunsaturated fatty acid (PUFA) biomarker depletion.

These data provide insight into sex-specific differences in antioxidant biomarker depletion and symptomology associated with a history of mTBI. These results also infer how symptom severity and frequency differ based on the number of mTBIs sustained and the time since a patient's last mTBI. Finally, our results support further exploration of antioxidant treatments, such as PUFA supplementation, to ameliorate the chronic neurological effects of mTBI.

Results

Patient population

Antioxidant biomarker levels measured by either Vibrant America Clinical Labs or Genova Diagnostics in whole blood, or components such as serum, plasma, RBCs, or WBCs, along with demographic information and medical history, were compiled from a total of 170 (n = 104 males, n = 66 females) patients who presented to Resilience Code, a specialized sports medicine clinic in Denver, CO. Blood samples and MSQ responses, which inquired about a variety of symptoms and their severities and frequencies, were taken at initial clinic visit

prior to treatment. A total of 88 patients reported sustaining at least one mTBI in their lifetime and were considered to have a history of mTBI whereas 82 patients never reported having a mTBI and therefore, were categorized as having no history of mTBI.

The average age of patients with mTBI was significantly lower ((38.95±1.81) years, p = 0.003) than those without a history of mTBI ((46.16±1.85) years). Male patients with a history of mTBI were driving this difference as they had a significantly lower average age ((37.55±2.09) y, n = 62, p = 0.001) than males without mTBI ((48.62±2.66) y, n = 42; Table 1). Average age was not significantly different between female patients with ((42.31±3.50) y, n = 26, p = 0.694) versus without a history of mTBI ((43.58±2.55) y, n = 40; Table 1). Age was not considered to be a confounding factor in the results to follow.

Patients with a history of mTBI were further divided into populations based on reported number of mTBIs sustained in their lifetime and time elapsed since their most recent mTBI (Table 2). Forty-six (n = 46) history of mTBI patients, including 29 males and 17 females, reported the cause of their most recent mTBI as sport, fall, car accident, or blunt injury and a significant association was found between sex and the cause of most recent mTBI (p < 0.001; Table 2). A flow diagram of patients and analyses performed is displayed in Figure S1.

Antioxidant depletion in patients with a history of mTBI when compared to those without a history of mTBI

Animal studies indicate that secondary injury resulting from mTBI may persist for months or longer following injury [28,29]. Therefore, we hypothesized that prolonged oxidative stress and inflammation post-mTBI may result in depletion of the endogenous antioxidant system. We aimed to fill a gap in the literature by analyzing differences in antioxidant biomarker levels between patients with and without a history of mTBI. Biomarker levels that were not significantly different or trending significantly different between the total patients with and without a history of mTBI are displayed in Table S1. All biomarkers were not measured in every patient; therefore, sample sizes for individual biomarkers vary as shown. In general, the differences in individual biomarker levels observed between patients with and without a history of mTBI, regardless of sex, were of small-to-medium effect size (Table S3).

Patients with a history of mTBI had significantly lower levels of alpha-tocopherol ((13.30±0.47) mg/L, n = 87, p = 0.004) and selenium ((137.32±2.80) ng/mL, p = 0.033, n = 48) in serum compared to patients without mTBI (alpha-tocopherol; (15.99±0.76) mg/L, n = 80; selenium; (155.98±7.66) ng/mL, n = 37; Fig. 1A, 1D). Patients with a history of mTBI also had significantly lower linoleic acid ((1125.74 ±26.08) μmol/L, n = 88, p = 0.003) and taurine ((43.69±1.72) μmol/L, n = 86, p < 0.001) in plasma when compared to patients without mTBI (linoleic acid; (1268.83±34.37) μmol/L, n = 81; taurine; (52.68±2.47) μmol/L, n = 77; Fig. 2B, 2C). Lastly, patients with a history of mTBI had significant decreases in docosahexaenoic acid ((6.47±0.30)%, n = 48, p = 0.042) and total omega-3 ((8.13±0.36)%, n = 48, p = 0.038) in RBCs compared to patients without mTBI (docosahexaenoic acid; (7.51 ±0.33)%, n = 37; total omega-3; (9.46±0.44)%, n = 37; Fig. 3A, 3B).

Extensive antioxidant depletion in male patients with a history of mTBI versus male patients without a history of mTBI

Increased oxidative stress markers are reported in healthy adult males compared to females [30,31]. Furthermore, females exhibit higher antioxidant capacity than males which may be due to sex hormones [32–34]. Males and females may have dissimilar antioxidant alterations in response to mTBI secondary pathology. To explore this, antioxidant biomarkers were compared between males and females with versus without a history of mTBI. Males with a history of mTBI displayed significantly or trending significantly decreased alpha-tocopherol ((12.88±0.54) mg/L, $n = 61$, $p = 0.002$) and selenium ((134.00 ±2.92) ng/mL, $n = 30$, $p = 0.072$) in serum compared to males without mTBI (alpha-tocopherol; (16.36±1.05) mg/L, $n = 41$; selenium; (145.42 ±4.87) ng/mL, $n = 16$; Fig. 1A, 1D). Males with a history of mTBI also had significant or trending significant decreases in plasma biomarkers such as linoleic acid ((1114.27±30.91) μmol/L, $n = 62$, $p = 0.072$) and taurine ((43.47±2.24) μmol/L, $n = 62$, $p = 0.005$) when compared to males without a history of mTBI (linoleic acid; (1244.27±53.49) μmol/L, $n = 41$; taurine; (52.73±4.22) μmol/L, $n = 40$; Fig. 2B, 2C). Lastly, males with a history of mTBI had significantly or trending significantly depleted levels of docosahexaenoic acid ((6.27±0.39)%, $n = 30$, $p = 0.068$) and total omega-3 ((7.83±0.43)%, $n = 30$, $p = 0.046$) when compared to males without mTBI (docosahexaenoic acid; (7.58 ±0.46)%, $n = 16$; total omega-3; (9.66±0.61)%, $n = 16$; Fig. 3A and 3B). All of these biomarkers noted in males were also significantly decreased in the total patients with versus without a history of mTBI. Interestingly, male patients with a history of mTBI also had additional significant or trending significant decreases in coenzyme-q10 ((1.27±0.07) mg/L, $n = 61$, $p = 0.051$) and cysteine ((23.25±2.31) nmol/mL, $n = 30$, $p = 0.094$) in serum, alpha-linolenic acid ((27.56±1.38) μmol/L, $n = 62$, $p = 0.088$) in plasma, and total omega-6 ((23.35±1.12)%, $n = 30$, $p = 0.035$) in RBCs when compared to males without mTBI (coenzyme-q10; (1.81 ±0.20) mg/L, $n = 41$; cysteine; (27.56±2.81) nmol/mL, $n = 16$; alpha-linolenic acid; (33.07±2.46) μmol/L, $n = 41$; total omega-6; (27.28±1.26)%, $n = 16$; Figs. 1B, 1C, 2A, and 3C).

In contrast to male patients with a history of mTBI who displayed widespread antioxidant biomarker depletion, female patients with a history of mTBI only had significant or trending significant decreases in linoleic acid ((1153.08±49.09) μmol/L, $n = 26$, $p = 0.053$) and taurine ((44.25±2.24) μmol/L, $n = 24$, $p = 0.017$) in plasma compared to females without mTBI (linoleic acid; (1294.00±43.21) μmol/L, $n = 40$; taurine; (52.62±2.43) μmol/L, $n = 37$; Fig. 2B, 2C).

Increased severity and frequency of emotional, energy, head, and cognitive symptoms in patients with a history of mTBI versus without a history of mTBI

Neuropsychological, emotional, and cognitive symptoms have been shown to persist for months to years following mTBI for some individuals [35,36]. The majority of our mTBI patient population presented to the clinic with persisting symptoms which were self-reported using the MSQ. We analyzed emotional, energy, head, and cognitive symptoms in patients with versus without a history of mTBI. For all symptom data, an increased score indicates more severe and frequent affective symptomology. MSQ symptom scores that were not significantly different or trending significantly different between the total patients with and

without a history of mTBI are displayed in Table S2. Some patients did not complete the MSQ or did not answer every question so n values for individual questions and scores vary as shown. In general, the differences in individual MSQ symptom scores observed between patients with and without a history of mTBI, regardless of sex, were of small-to-medium effect size (Table S3).

Patients with a history of mTBI had a trending significantly greater total symptom score (54.81±3.87, $n = 69$, $p = 0.069$) when compared to patients without mTBI (45.48±3.78, $n = 64$; data not shown). Total patients with a history of mTBI had a significantly increased emotions total score (5.29±0.49, $n = 69$, $p = 0.016$) when compared to patients without mTBI (3.58±0.38, $n = 64$; Fig. 4A). Significantly increased emotional symptoms included anxiety/fear (1.65±0.17, $n = 69$, $p = 0.049$), depression (1.30±0.16, $n = 69$, $p = 0.024$), and mood swings (1.20±0.13, $n = 69$, $p = 0.019$) when compared to patients without mTBI (anxiety/fear; 1.14±0.14, $n = 64$; depression; 0.78±0.12, $n = 64$; mood swings; 0.80±0.10, $n = 64$; Fig. 4B–D). A significantly greater energy total symptom score was also observed in patients with a history of mTBI (5.14±0.51, $n = 69$, $p = 0.046$) versus patients without mTBI (3.44±0.34, $n = 64$, Fig. 5A). Significantly or trending significantly increased energy symptoms in patients with a history of mTBI included apathy/lethargy (1.38±0.18, $n = 69$, $p = 0.026$), hyperactivity (0.62 ±0.13, $n = 68$, $p = 0.060$), and restlessness (1.20±0.15, $n = 69$, $p = 0.006$) when compared to patients without mTBI (apathy/ lethargy; 0.80±0.15, $n = 64$; hyperactivity; 0.30±0.09, $n = 64$; restlessness; 0.63 ±0.10, $n = 64$, Fig. 5B, D, and E). For head and cognitive symptoms, history of mTBI patients only had trending significantly increased scores for faintness (0.59±0.12, $n = 69$, $p = 0.072$) and confusion (0.80±0.14, $n = 69$, $p = 0.055$), respectively, when compared to patients without mTBI (faintness; 0.31±0.09, $n = 64$; confusion; 0.52±0.13, $n = 64$; Fig. 6B, 7B).

Male patients with a history of mTBI exhibited overall increased symptom severity and frequency for emotional, head, and cognitive symptoms

Previous literature has reported differences in male and female patient outcomes, which includes symptomology, following mTBI [37,38]. Therefore, symptom severity and frequency for emotion, energy, head, and cognitive symptoms were also analyzed for male and female patients with and without a history of mTBI to determine if sex-specific differences existed. As indicated above, for all symptom data, an increased score indicates more severe and frequent affective symptomology.

Parallel to the total mTBI patients, males with a history of mTBI had a significantly increased emotions total score (4.89±0.63, $n = 45$, $p = 0.024$) when compared to males without mTBI (2.66±0.47, $n = 32$; Fig. 4A). Significantly or trending significantly increased emotional symptoms in males with a history of mTBI included anxiety/fear (1.47 ±0.20, $n = 45$, $p = 0.016$), depression (1.18±0.20, $n = 45$, $p = 0.089$), and mood swings (1.20±0.16, $n = 45$, $p = 0.007$) when compared to males without mTBI (anxiety/fear; 0.78±0.19, $n = 32$; depression; 0.66 ±0.19, $n = 32$; mood swings; 0.56±0.12, $n = 32$; Fig. 4B– D). For energy symptoms, in contrast to the total history of mTBI patients, males with a history of mTBI only had significantly or trending significantly increased scores for hyperactivity (0.50±0.16, $n = 44$, $p = 0.073$) and restlessness (1.04±0.16, $n = 45$, $p = 0.014$) when compared to

males without mTBI (hyperactivity; 0.16±0.07, $n = 32$; restlessness; 0.47±0.10, $n = 32$; Fig. 5D and 5E). Male patients with a history of mTBI had a significantly increased head symptom total score (3.07±0.46, $n = 45$, $p = 0.029$) including significantly or trending significantly increases in faintness (0.42±0.11, $n = 45$, $p = 0.012$), headaches (0.91±0.20, $n = 45$, $p = 0.060$), and insomnia (1.38±0.23, $n = 45$, $p = 0.035$), when compared to males without mTBI (total head score; 1.44±0.27, $n = 32$; faintness; 0.06±0.04, $n = 32$; headaches; 0.38±0.13, $n = 32$; insomnia; 0.65 ±0.18, $n = 31$; Fig. 6A– 6D). Similar to the total history of mTBI population, males with history of mTBI did not have a significantly increased or trending significantly increased total cognitive score (4.16 ±0.73, $n = 45$, $p = 0.199$) but did have a trending significantly increased symptom score for confusion (0.64±0.16, $n = 45$, $p = 0.077$) compared to males without mTBI (total cognitive score; 2.84±0.71, $n = 32$; confusion; 0.38±0.18, $n = 32$; Fig. 7A and 7B). History of mTBI males had additional significant or trending significant increases in cognitive symptom scores for difficulty making decisions (0.73±0.16, $n = 45$, $p = 0.065$), slurred speech (0.13±0.08, $n = 45$, $p = 0.085$), and stuttering (0.20±0.06, $n = 45$, $p = 0.091$) versus males without mTBI (difficulty making decisions; 0.41±0.18, $n = 32$; slurred speech; 0.00±0.00, $n = 32$; stuttering; 0.06±0.04, $n = 32$; Fig. 7C, 7E, and 7F).

Female patients with a history of mTBI showed increases in symptom severity and frequency for distinct energy symptoms

For emotion, females with history of mTBI displayed a trending significantly increased emotions total score (6.04±0.076, $n = 24$, $p = 0.059$) compared to females without mTBI (4.50±0.56, $n = 32$; Fig. 4A). In addition, females with history of mTBI had significantly increased scores for depression (1.54±0.26, $n = 24$, $p = 0.039$) when compared to females without mTBI (0.91±0.16, $n = 32$; Fig. 4C). For energy, while mTBI males only had significantly or trending significantly increased scores for hyperactivity and restlessness, female mTBI patients showed a significant increase in total energy score (7.13±0.89, $n = 24$, $p = 0.016$) which included significant or trending significant increases for apathy/lethargy (2.17±0.33, $n = 24$, $p = 0.005$), fatigue (2.79±0.29, $n = 24$, $p = 0.085$), and restlessness (1.50±0.30, $n = 24$, $p = 0.081$) versus females without mTBI (total energy score; 4.34±0.55, $n = 32$; apathy/lethargy; 1.00±0.23, $n = 32$; fatigue; 2.13±0.26, $n = 32$; restlessness; 0.78±0.17, $n = 32$; Fig. 5A– 5E). In contrast to males with mTBI, females with a history of mTBI had no head symptoms that were significantly, or trending significantly increased compared to females without mTBI (Fig. 6). Furthermore, females with a history of mTBI only had a trending significantly increased score for poor concentration (1.96 ±0.32, $n = 24$, $p = 0.061$) for cognitive symptoms when compared to females without mTBI (1.22±0.23, $n = 32$; Fig. 7D). These data indicate that males exhibit an overall increased symptom severity and frequency for a diverse array of emotional, head, and cognitive symptoms; however, female patients show increases in symptom severity and frequency for specific energy symptoms in response to mTBI (e.g., apathy/lethargy and fatigue for females versus hyperactivity for males).

Multiple mTBI patients reported worsened and more frequent symptomology compared to patients who sustained a single mTBI

Sixty-seven (n = 67) of the 88 history of mTBI patients reported either sustaining a past single mTBI (n = 27) or multiple mTBIs (n = 40). A patient was included in the multiple mTBI group if they sustained more than one mTBI throughout their lifetime, regardless of time elapsed between mTBIs. Previous data indicates that patients who suffer multiple mTBIs may have exacerbated secondary pathology which could lead to worsened symptoms [9,10]. Therefore, a qualitative heatmap visualization was created to allow for a comparison of increased symptom severity and frequency for the previously mentioned emotional, energy, head, and cognitive symptoms in patients with multiple mTBIs when compared to patients with a past single mTBI and no mTBI (Fig. 8A). Patients with multiple mTBIs seem to have worsened symptom severity and frequency, as indicated by the darker shade of grey for symptom scores, when compared to patients with a past single mTBI or no history of mTBI.

Chronic mTBI patients reported worsened and more frequent emotional, energy, and cognitive, but not head symptoms, when compared to acute/subchronic mTBI patients

We also wanted to know whether symptom severity and frequency changed with increased time elapsed since the mTBI. History of mTBI patients were divided into groups based on the amount of time that has passed since their most recent mTBI to the clinic visit. Of the 88 history of mTBI patients, 46 reported the date of their most recent mTBI. Fourteen (n = 14) patients suffered acute/subchronic mTBI and presented to the clinic within 1 year of their most recent mTBI (6.80±1.19 months; range = 0.2–12 months). Thirty-two (n = 32) patients suffered chronic mTBI with their most recent mTBI > 1 year prior to presenting to the clinic (12.29±2.17 years; range = 1.42–40 years). Emotional, energy, head, and cognitive symptom scores between no, acute/subchronic, and chronic mTBI patients were also qualitatively analyzed using a heatmap visualization (Fig. 8B). As indicated by a darker grey shade, it appears that patients with chronic mTBI have worsened and more frequent symptoms in most all categories aside from hyperactivity and most head symptoms when compared to acute/subchronic and no mTBI patients.

History of mTBI patients with more depleted levels of PUFA biomarkers have significantly worsened and increased frequency of symptoms when compared to mTBI patients with less depleted levels of PUFA biomarkers

Supplementation with PUFAs prior to and following TBI have been shown to reduce neuronal cell death, inflammation, and oxidative stress and improve cognitive function [39–42]. We hypothesized that higher PUFA levels could result in improved outcome and less severe symptomology in humans following TBI. Our data indicate that PUFAs such as total omega-3, total omega-6, linoleic acid, alpha-linolenic acid, and docosahexaenoic acid are chronically depleted with mTBI. Therefore, we suspected that greater depletion in these antioxidants may be responsible for worsened symptomology in patients with a history of mTBI.

Patients with a history of mTBI that had biomarker data for all PUFAs analyzed (linoleic acid and alpha-linolenic acid in plasma and total omega-3, total omega-6, and

docosahexaenoic acid in RBCs) and MSQ responses ($n = 43$) were divided into patients with a low ($n = 20$) and high level of PUFAs ($n = 23$). History of mTBI patients with a low level of PUFAs had a significantly lower level of PUFAs (9.15±0.47, $p < 0.000$) when compared to mTBI patients with a high level (13.96±0.42, Fig. 9A). Overall, history of mTBI patients with a low PUFA score had a significantly higher total symptom score (65.15±6.46), indicating worsened symptom severity and increased frequency, when compared to patients with a higher PUFA score (47.39±6.34, $p = 0.028$, Table S4). Emotional, energy, head, and cognitive symptom scores were analyzed between these patients. Of these, the energy total symptom score was significantly increased in history of mTBI patients with low levels of PUFAs (6.65±0.86) versus those with higher levels (3.96±0.81, $p = 0.022$, Fig. 9B).

History of mTBI patients with low PUFA levels had significantly increased scores for energy symptoms for fatigue (2.60±0.31, $p = 0.038$), apathy/lethargy (1.80±0.31, $p = 0.035$), and restlessness (1.60 ±0.27, $p = 0.023$) when compared to patients with high levels (fatigue; 1.70±0.29; apathy/lethargy; 1.09±0.33; restlessness; 0.91±0.26; Fig. 9C). Patients with a history of mTBI with low levels of PUFAs also had significantly increased symptom scores for anger/irritability (1.75 ±0.28, $p = 0.018$) for emotional symptoms and confusion (1.50±0.34, $p = 0.023$) for cognitive symptoms when compared to patients with high levels of PUFAs (anger/irritability; 0.87±0.16; confusion; 0.48±0.17; Fig. 9C). In general, the differences in symptom scores observed between mTBI patients with low vs high PUFA levels were of small-to-medium effect size (Table S4). These data indicate that mTBI patients with lower levels (i.e., more depleted) of PUFA biomarkers have worsened symptomology when compared to patients with higher levels (i.e., less depleted) of PUFAs.

Male history of mTBI patients with more severe depletion of PUFA biomarkers have worsened symptomology when compared to male mTBI patients with higher levels of PUFA biomarkers

Since we observed more widespread antioxidant depletion in males with a history of mTBI and sex-specific differences in symptoms, we hypothesized that PUFA antioxidant depletion and subsequent worsened symptomology was also sex-specific. Therefore, male patients with a history of mTBI that had biomarker data for all PUFAs analyzed and MSQ responses ($n = 26$) were divided into patients with a low ($n = 12$) and high level of PUFAs ($n = 14$). Male history of mTBI patients with a low level of PUFAs had a significantly lower level of PUFAs (9.17±0.67, $p < 0.001$) when compared to male mTBI patients with a high level (14.14±0.62, Fig. 10A). Male history of mTBI patients with a low PUFA score had a significantly higher total symptom score (60.50±7.65), indicating worsened symptom severity and increased frequency, when compared to patients with a higher PUFA score (37.07±6.26, $p = 0.004$, Table S5. Emotional, energy, head, and cognitive symptom scores were analyzed between these male patients. Like what was observed in the total history of mTBI population, the energy total symptom score was significantly increased in male history of mTBI patients with low levels of PUFAs (5.83±0.88) versus those with higher levels (2.79±0.81, $p = 0.013$, Fig. 10B).

Male history of mTBI patients with low PUFA levels had significantly increased scores for energy symptoms for fatigue (2.25±0.39, $p = 0.046$) and apathy/lethargy (1.50±0.34, $p =$

0.017) and a trending significantly increased score for restlessness (1.33±0.19, $p = 0.067$) when compared to male patients with high levels (fatigue; 1.21±0.26; apathy/lethargy; 0.50±0.25; restlessness; 0.86±0.29; Fig. 10C). Male patients with a history of mTBI with low levels of PUFAs also had significantly increased symptom scores for anger/irritability (1.67±0.36, $p = 0.031$) for emotional symptoms and a trending significantly increased score for confusion (1.50±0.47, $p = 0.060$) for cognitive symptoms when compared to male patients with high levels of PUFAs (anger/irritability; 0.64±0.20; confusion; 0.29±0.16; Fig. 10C). In general, the differences in symptom scores observed between male mTBI patients with low vs high PUFA levels were of small-to-medium effect size (Table S5). These symptoms were also observed to be significantly increased in the total history of mTBI patients with lower levels of PUFA biomarkers.

Interestingly, although females with a history of mTBI with low PUFA levels ($n = 8$) did have a significantly lower level of PUFAs (9.13 ±0.64, $p < 0.001$) when compared to female mTBI patients with a high level ($n = 9$; 13.67±0.47), they did not display any significant or trending increased scores for total, emotional, energy, head, or cognitive symptoms when compared to female mTBI patients with higher level of PUFAs. Furthermore, individual symptom scores for these symptom categories were not significantly or trending increased in female history of mTBI patients with low versus high PUFA scores. Taken together, these data suggest that male patients with a history of mTBI respond uniquely worse to PUFA antioxidant depletion than females, resulting in more severe and frequent symptoms.

Discussion

In the current study, we retrospectively analyzed antioxidant biomarkers and symptomology in patient cohorts with and without a history of mTBI from a local sports medicine clinic in Denver, CO. We found that the total population with a history of mTBI had significantly depleted levels of biomarker antioxidants including alpha-tocopherol and selenium in serum, linoleic acid and taurine in plasma, and docosahexaenoic acid and total omega-3 in RBCs when compared to patients without mTBI. Male patients with a history of mTBI, compared to males without mTBI, showed significant or trending significant depletions in all antioxidant biomarkers that were depleted in the total mTBI population. Furthermore, male patients with a history of mTBI had additional significant or trending significant decreases in coenzyme-q10 and cysteine in serum, alpha-linolenic acid in plasma, and total omega-6 in RBCs when compared to males without mTBI. On the other hand, female mTBI patients only showed significantly or trending significantly decreased linoleic acid and taurine in plasma, which were depleted in the total population with a history of mTBI, when compared to females without mTBI. Overall, patients with a history of mTBI display significant and chronic antioxidant biomarker depletion and male patients show more extensive antioxidant depletion than female patients in response to mTBI.

The secondary injury pathology response to TBI, even of mild severity, can include neuroinflammation, oxidative stress, BBB disruption, mitochondrial dysfunction, and excitotoxicity. Previous research has shown that this pathology can persist for weeks to months following mTBI [3–6]. Neuroinflammation consists of infiltrating macrophages as a result of BBB disruption and activated resident microglia and astrocytes which generate

ROS/RNS and contribute to the oxidative burden [19]. Excessive glutamate release and subsequent calcium overload results in excitotoxicity. Excitotoxicity, along with increased oxidative stress, can initiate apoptotic signaling pathways within mitochondria leading to neuronal cell death [18]. When these processes persist, the endogenous antioxidant system becomes depleted as brain endogenous antioxidants like superoxide dismutase (SOD) or glutathione (GSH) work to remove and neutralize ROS/RNS. Only a few studies have explored antioxidant depletion following TBI [26,27,43,44]. However, to our knowledge, no studies to date have reported extensive and chronic antioxidant depletion in human subjects in response to mTBI. The data presented here are novel in that they confirm significant and prolonged antioxidant depletion in patients with a history of mTBI.

We hypothesized that we would observe sex-specific differences in antioxidant depletion in response to a history of mTBI, with male patients having more severe antioxidant depletion and resulting worsened symptom severity and frequency. In the current study, we found that male patients exhibit more extensive antioxidant depletion than females in response to mTBI. Previous studies have shown that male subjects have increased ROS/RNS species, increased markers of oxidative damage such as thiobarbituric acid-reactive substances (e.g., malondialde-hyde), and reduced antioxidants such as SOD, GSH peroxidase, and catalase, when compared to females [30,31,45]. Total antioxidant capacity is also higher in females [32–34]. In response to TBI of greater severity and in animal studies of TBI, oxidative stress, as evidenced by lipid peroxidation and protein carbonylation, was increased in males with TBI [46–48]. In disease and TBI, female sex hormones such as 17β-estradiol and progesterone, are neuroprotective and play a role in increasing activity of the endogenous antioxidant system [33,45,49]. Furthermore, studies have shown that female sex hormones are neuroprotective against TBI [50,51]. In fact, progesterone has been explored as a potential therapeutic for TBI with conflicting results. Although factors such as differing treatment protocols and healthcare conditions confound the data from these studies, these conflicting results may also be indicative of complex interactions of the sex hormones with the endogenous antioxidant system. [52,53]. Since healthy males already have an increased oxidative burden and reduced antioxidant potential, along with a lack of neuroprotective sex hormones, our findings support the view that in response to the overt and prolonged oxidative stress caused by a history of mTBI, males display more extensive and persistent antioxidant depletion than females.

Severity and frequency of emotional, energy, head, and cognitive symptoms were also analyzed in total, male, and female populations with a history of mTBI. Total patients with mTBI had significant or trending significant increases in the following symptoms: total emotion symptom score which included anxiety/fear, depression, and mood swings; total energy symptom score which included apathy/lethargy, hyperactivity, and restlessness; only faintness for head symptoms; and no significant increase in cognitive symptom scores. Males and females exhibited sex-specific differences in trending or significantly increased symptom scores. Male patients with a history of mTBI had worsened symptom severity and frequency for most symptoms, particularly for head and cognitive symptoms, whereas females exhibited less overall symptomology but more severe symptom severity and frequency for energy symptoms. These sex-specific findings in symptoms following mTBI are supported by preclinical research stating that males exhibit worsened cognitive

symptoms, including memory deficits, following mTBI [54,55]. These findings are further supported by clinical data that also suggest sex-specific differences in cognitive functioning, with males exhibiting worsened memory and language skills, following TBI [56,57]. Sustained increased oxidative burden leading to extensive antioxidant depletion is a likely contributor to physical symptoms. Many of the antioxidants observed to be depleted in our history of mTBI patients are known not only to reduce oxidative stress, but also act as neuroprotective agents and have anti-inflammatory properties. When depleted long-term, physical symptoms may manifest and antioxidant depletion may contribute to the development of neurodegenerative disease.

Patients with a history of mTBI were further divided based on number of mTBIs sustained and time since their last mTBI. Qualitative analysis indicated that multiple mTBI patients had worsened symptom scores for all categories when compared to single mTBI patients and patients without mTBI. Multiple mTBIs can exacerbate secondary injury and healthy adults who have rmTBIs report worse cognitive and emotional symptoms [8,9]. Chronic mTBI patients also showed worsened symptom scores for emotional, energy, and cognitive symptoms when compared to acute/subchronic mTBI patients and patients without mTBI. Interestingly, acute/subchronic mTBI patients had worsened symptoms for hyperactivity and most head symptoms. Persistent symptoms from mTBI occur in 10–15 % of patients which typically include headache, sleep disturbances, and fatigue [58]. Patients with persisting symptoms likely represent our chronic mTBI patients as they presented to the clinic with continuing symptoms greater than 1 year since their last mTBI.

Alpha-tocopherol has implications in nerve protection and cognitive function, resulting in neurological symptoms when depleted [59]. Selenium is a trace mineral that plays roles in the formation of seleno-proteins, including GSH peroxidase, which performs antioxidant activities, and its depletion has been linked to immune dysfunction, decreased neurotransmitter production, and depressed mood states [60]. Both alpha-tocopherol and selenium were significantly, or trending significantly, depleted in total and male patients with a history of mTBI which may explain the worsened head and cognitive symptoms reported particularly by male patients with mTBI.

Taurine, which was depleted in all history of mTBI populations, is a sulfur-containing amino acid that has anti-inflammatory roles, can reduce superoxide generation, prevents apoptosis, supports energy metabolism, and protects against excitotoxicity [61,62]. Depletion of taurine can result in depression and anxiety, symptoms that were significantly or trending significantly worsened in all history of mTBI populations.

Taurine is synthesized from cysteine. Although taurine was found to be depleted in all mTBI populations, cysteine was only found to be trending significantly depleted in males with a history of mTBI. Cysteine is an amino acid necessary for protein synthesis, a precursor to many sulfur containing molecules, and is necessary for GSH synthesis. Cysteine plays roles in redox homeostasis and has implications for numerous neurodegenerative diseases [63].

Coenzyme-q10 was trending significantly depleted in males with mTBI and is a component of the mitochondrial electron transport chain. It has roles in energy metabolism and

mitochondrial function, with implications in neurodegenerative disease [64]. Males had deficiencies in many of the antioxidant biomarkers with implications in neurodegenerative disease and also had increased symptomology for head and cognitive symptoms. The connection between antioxidant depletion in response to mTBI and neurodegenerative disease should be further explored.

Polyunsaturated fatty acids found to be depleted in the mTBI population in this study include linoleic acid, alpha-linolenic acid, total omega-3, total omega-6, and docosahexaenoic acid. Polyunsaturated fatty acids provide structure and support to both neurons and glia [65]. The brain is particularly enriched in arachidonic acid and docosahexaenoic acid, which were measured in this study. We found that mTBI patients with lower levels of PUFAs had significantly worsened anger/irritability for emotional symptoms, confusion for cognitive symptoms, and energy total score which included significantly worsened fatigue, apathy/lethargy, and restlessness. Interestingly, when we analyzed sex-specific differences in symptom responses to PUFA depletion, we found that males with significantly lower levels of PUFAs had significantly or trending significantly worsened symptoms that were the same as observed in the total population. In contrast, no significant differences were observed in females with lower levels of PUFAs. These findings support previous findings within this publication which suggest that males respond with worsened symptoms to antioxidant depletion following mTBI. Depletions in PUFAs, particularly docosahexaenoic acid, have been associated with mood changes including depression and bipolar disorder [66]. Male mTBI patients had significantly or trending significantly decreased levels of linoleic acid, alpha-linolenic acid, total omega-3, total omega-6, and docosahexaenoic acid when compared to male patients without mTBI, which coincides with their reported worsened emotional, head, and cognitive symptoms. Polyunsaturated fatty acids have been well studied in vivo models as treatments for TBI. Supplementation with PUFAs, such as docosahexaenoic acid and omega-3, result in neuroprotection and decreased inflammation and oxidative stress following TBI [39–42]. Furthermore, PUFA supplementation can inhibit microglial activation, suppress necroptosis, and preserve brain derived neurotrophic factor levels in animal models of mTBI [67–70]. These data add further support to the idea of supplementing the diet with PUFAs, to treat not only the acute but also the chronic effects of mTBI.

There are several limitations to the present study. Due to the retrospective nature of this study, we could not collect additional information for participants who were missing information resulting in varying n values for biomarker levels and symptom scores. We also found that the average age of patients with mTBI was significantly lower than patients without a history of mTBI, with males driving this difference. Research has shown that antioxidant deficiency occurs in individuals aged 65 years and older [71,72]. Our study populations with and without a history of mTBI had an average age that is well below 65 years. Furthermore, increased oxidative stress and inflammation, which could result in antioxidant depletion, occur following TBI [18,19]. Our history of mTBI population is significantly younger than the population without mTBI and we would expect these younger patients to have more robust antioxidant systems. Thus, findings of antioxidant depletion in these younger patients in response to mTBI would be even more compelling than if observed in an older cohort. Also, due to the retrospective nature of this study, patients presented

to the clinic within a wide range of time elapsed since their most recent mTBI or didn't report the date of their last mTBI. We recognize that there may not be a direct correlation between antioxidant depletion and the presentation of certain symptoms and that other biochemical changes, environmental exposures, and social factors may also contribute to the reported symptoms observed. As previous studies highlight, these factors may also play a role in the sex-specific differences in outcomes following TBI [73]. Future studies should include larger sample sizes, complete medical history and life-style information, thorough information pertaining to the nature of the mTBI along with biomarker measurements, and symptom responses ideally at regular intervals from the first mTBI. However, this study is valuable in that it is the first of its kind to analyze numerous antioxidant biomarkers along with a comprehensive list of symptoms in male and female patients with a history of mTBI.

Conclusion

The data presented here show that overall, patients with a history of mTBI have significant and prolonged antioxidant biomarker depletion and worsened symptomology than patients without a history of mTBI. Furthermore, male patients were observed to have more extensive antioxidant depletion and worsened symptom severity and frequency for most symptoms, especially head and cognitive symptoms. Males with a history of mTBI that had lower levels of PUFA antioxidants also reported worsened symptoms. On the other hand, females with history of mTBI had less overall antioxidant depletion and symptom severity/frequency. In contrast to males, females with a history of mTBI also showed no worsened symptomology with more severe PUFA depletion. The data support the use of antioxidant supplementation (e.g., PUFAs) following mTBI, particularly in patients with chronic symptoms and those who have suffered from multiple mTBIs, as a therapeutic approach to mitigate the long-term adverse effects caused by mTBI.

Methods

Participant population

Data from 170 patients admitted to a sports medicine clinic between 11/2017–9/2020, (Resilience Code; Englewood, Colorado) were retrospectively analyzed for this study. Patients were evaluated by a board-certified physician who collected demographic information and medical history. Patients also completed the MSQ and provided blood for analysis of biomarkers (detailed below) prior to the administration of any treatment. Patients with active cancer at the time of visit were excluded. A total of 88 patients, including 62 males and 26 females, having at least one traumatic brain injury (TBI) in their lifetime, were considered to have a history of TBI based on patient reporting and medical history. The board-certified physician confirmed all history of TBI patients as having TBIs of mild severity. The 88 patients with a history of mTBI were further categorized by number of mTBIs sustained throughout their lifetime and time elapsed since their most recent mTBI to the clinic visit. A total of 27 patients reported sustaining a single mTBI, 40 sustained multiple mTBIs, and 21 patients did not report this information and were not included in analyses of these populations. A patient was included in the multiple mTBI group if they sustained more than one mTBI throughout their lifetime, regardless of time elapsed between

mTBIs. Of the 88 history of mTBI patients, 14 patients presented to the clinic within 1 year of their most recent mTBI, termed as acute/subchronic mTBI patients, and 32 patients presented to the clinic greater than 1 year since their most recent TBI, termed chronic mTBI patients. A total of 42 patients did not report this information and were not included in analyses of these populations. Of the 170 total patients, 82 patients, consisting of 42 males and 40 females, never reported having a mTBI in their lifetime and were considered to have no history of mTBI. Demographic information for these populations is displayed in Tables 1 and 2. A flow diagram of patients and analyses performed are displayed in Figure S1.

Blood collection, processing, and analysis

Blood samples were obtained from all 170 patients. However, n values for each biomarker analyzed in blood vary and are reported for each individual biomarker as blood from each patient was not analyzed for every biomarker. Blood samples were obtained by a certified phlebotomist at Resilience Code and were processed according to manufacturer's protocols to isolate serum, plasma, RBCs, or WBCs. Blood was stored at Resilience Code at 4 °C for no longer than 8 h prior to being shipped to Vibrant America Clinical Labs (San Carlos, CA) or Genova Diagnostics (Asheville, North Carolina) for analysis of biomarkers in serum, plasma, RBC, WBCs, or whole blood.

Medical symptom questionnaire

The presence, frequency, and severity of symptoms at the time of the clinic visit were gathered using a 71-item electronic MSQ that took an average of 15 min to complete. A modified MSQ was used as previously described [74]. Of the total 170 patients, 139 patients completed the MSQ. A total of 69 of the 88 patients with a history of TBI, consisting of 45 males and 24 females, completed the MSQ. A total of 64 out of 82 patients without a history of TBI, including 32 males and 32 females, completed the MSQ. Some patients did not complete every question on the MSQ so n values for individual symptoms vary and are reported. The presence of specific symptoms and symptom severity were reported on a 4-point Likert scale with 0 indicating never or almost never having the specific symptom, 1 being occasionally having the symptom but the effect is not severe, and 4 being frequently having the symptom and the effect is severe. Symptom categories pertain to weight, skin, nose, mouth, cognition, chest, joints, heart, head, eyes, energy, emotions, ears, digestion, and other. Each individual symptom falls under one of these 15 categories and a total score for each symptom category is calculated for each patient by adding up the scores for the individual symptoms under the category. A total symptom score is also calculated for each patient by adding up all scores for each symptom category. A higher symptom category or total symptom score indicates increased symptom severity and frequency of symptoms. A total symptom score of 0 is the lowest score possible and a score of 284 is the highest score possible.

Statistics

All data was analyzed using SPSS Statistical Software (IBM Corp., Version 25, Armonk, NY) and graphs and heatmap visualizations were created using R Studio (R Studio Team [2021]. rStudio: Integrated Development for R. rStudio, Inc., Boston, MA) for Windows. Antioxidant blood biomarker and MSQ data for total, male, and female patients without

versus with a history of mTBI and for history of mTBI patients with levels of high versus low PUFA biomarkers are displayed graphically as boxplots using R Studio ggplot2 package and geom_boxplot function [75]. Boxplot components are displayed as follows: lower whisker = smallest observation greater than or equal to lower hinge-1.5 * interquartile range (IQR), lower hinge = 25 % quantile, middle line = median at 50 % quantile, diamond = mean, upper hinge = 75 % quantile, upper whisker = largest observation less than or equal to upper hinge+1.5 * IQR, outlying points = data beyond end of upper or lower whiskers [76]. Due to the clinical nature of the data, which was mostly non-normally distributed with unequal variances, as analyzed by Shapiro-Wilk and Levene's tests, respectively, nonparametric statistical tests were used to analyze all data (detailed below). Only one value for taurine in plasma was excluded from analysis from a female patient with a history of mTBI as this value was an extreme outlier. No other values were excluded. Statistical differences were considered significant if $p < 0.05$ and trending significant if $p < 0.10$. For significant or trending significant data analyzed by the Mann Whitney U-Test (detailed below), the Wilcoxon effect sizes were calculated based on the absolute value of the Z statistic (Z) and number of cases (N) using the formula $r = Z/\sqrt{N}$. Effect sizes are interpreted as follows: 0.10 - < 0.3 = small effect, 0.30 - < 0.5 = moderate effect, and 0.5 and greater = large effect [77]. All de-identified data was compiled and checked by at least two separate researchers. Data was also analyzed and checked by two separate researchers.

Demographic analysis of total, male, and female patients without versus with a history of mTBI

The Mann Whitney U-Test was used to analyze differences in age between total, male, and female patients without versus with a history of mTBI and in patients. The association of history of mTBI or reported cause of most recent mTBI and sex was analyzed using the Pearson Chi Square Test.

Analysis of antioxidant blood biomarker levels and MSQ data from total, male, and female patients without versus with a history of mTBI

Blood biomarker levels and MSQ symptoms pertaining to emotional state, energy, head, and cognition in total patients without a history of mTBI versus with a history of mTBI, male patients without a history of mTBI versus with a history of mTBI, female patients without a history of mTBI versus with a history of mTBI were analyzed using the nonparametric Mann Whitney U-Test.

Heatmap analysis of MSQ data from single, multiple, acute, chronic, and no history of mTBI patients

N values were too low for quantitative analysis of blood biomarkers levels and MSQ data in patients with a single mTBI versus a history of multiple mTBI or between patients with acute/subchronic mTBI versus chronic mTBI. Therefore, differences in MSQ scores between these groups pertaining to emotional state, energy, head, and cognition were visualized using a heatmap using R Studio ggplot2 package and geom_tile function [75]. Briefly, MSQ scores were converted to z scores to allow for normalization of individual symptom scores and symptom category scores, which exhibit different scoring ranges, for patients with single mTBI, multiple mTBI, and no history of mTBI. This was also

done for patients with acute/subchronic mTBI, chronic mTBI, and no history of mTBI to allow for symptom severity comparison between these groupings. The z score average was calculated for each individual symptom/symptom total category score. An increased z score corresponds to an increased symptom score indicating a severe and frequent symptom. Each row represents an individual symptom/symptom category total, and each column represents the average score for the different populations. Rows and columns were not clustered. The lightest grey shade corresponds to a z score of −0.30 and the darkest shade corresponds to 0.40 for single, multiple, no mTBI patients and to −0.30 and 0.80, respectively, for no, acute, and chronic, mTBI patients.

Analysis of MSQ data from total, male, and female patients with a history of mTBI with high versus low levels of PUFA biomarkers

Blood biomarker levels for alpha-linolenic acid and linoleic acid in plasma and total omega-6, total omega-3, and docosahexaenoic acid in RBCs, termed a PUFA biomarker grouping, were divided into quartiles for total, male, and female history of mTBI patients. A score of 1–4, corresponding to the quartile the patient's blood biomarker level was in (1 being the lowest quartile and 4 being the highest quartile), was given for each biomarker for each history of mTBI patient who had measurements for all PUFA biomarkers listed above. Quartile scores for the PUFA biomarkers were added for each patient and the patient was given a total PUFA score which had a possible range of 5–19. Patients with high versus low PUFA scores were divided into two groups based on a median split (median=12). Patients with a score of 11 or less (total: $n = 20$, range = 5–11; males: $n = 12$, range = 5–11; females: $n = 8$, range = 6–11) were considered to have a low PUFA score (i.e., PUFA depletion) and patients with a score of 12 or more (total: $n = 23$, range=12–19; males: $n = 14$, range = 12–19; females: $n = 9$, range =12–16) were considered to have a high PUFA score. The PUFA biomarker score for total, male, and female patients with low versus high PUFA scores were significantly different as analyzed the Mann Whitney U-Test. Medical symptom questionnaire scores in total, male, and female history of mTBI patients with high versus PUFAs were analyzed by the Mann Whitney U-Test.

Study approval

All patients granted Resilience Code specific, written authorization to disclose their medical records for research purposes. This study was retrospective and all protected health information was de-identified prior to compilation of data and analysis. Each patient was assigned a random global unified identifier which was used on all reports and forms associated with the study to maintain anonymity. Therefore, this study was determined to be exempt from The University of Denver's Institutional Review Board.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Data availability

Data will be made available on request.

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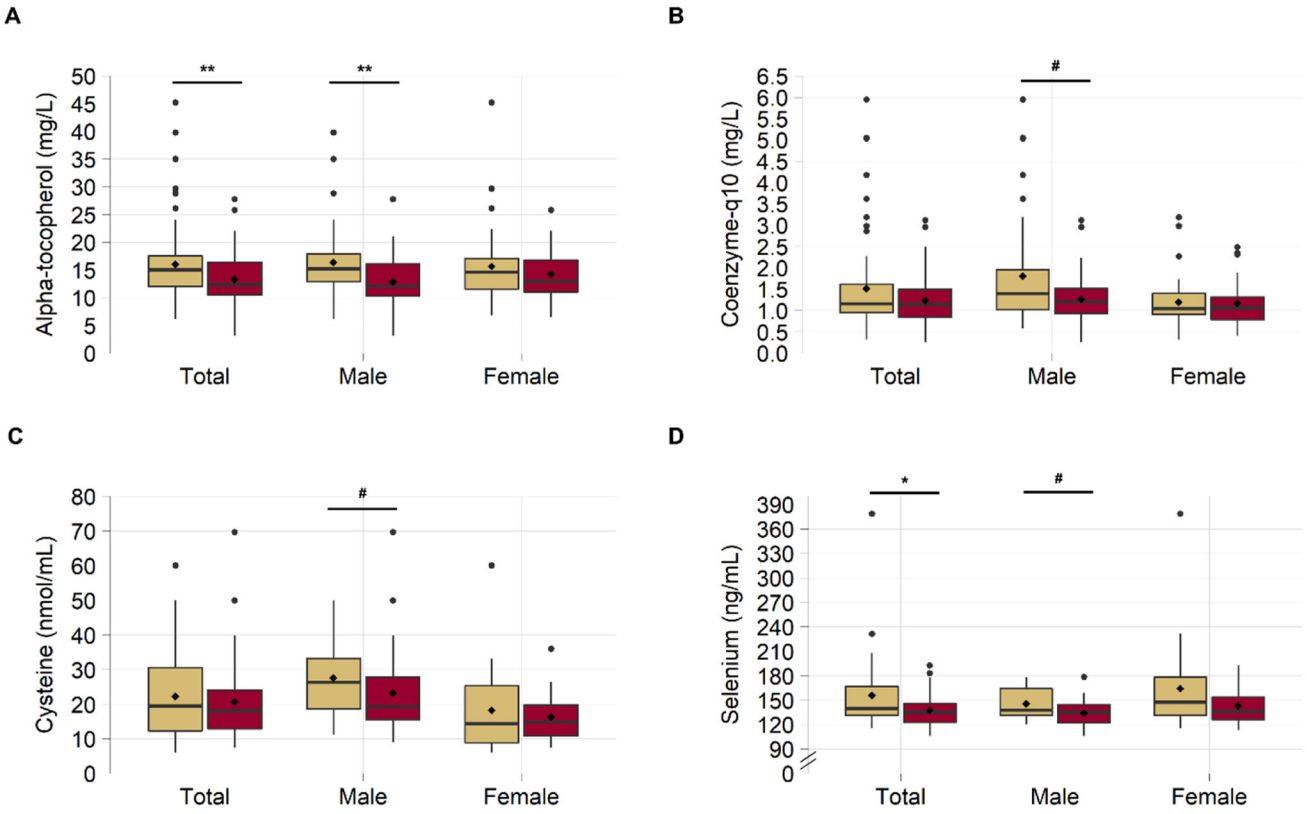

Fig. 1. Significantly or trending significantly reduced levels of serum antioxidant biomarkers in total, male, and female patients without versus with a history of mTBI.

In each graph, the gold plot represents populations without a history of mTBI and the crimson plot represents populations with a history of mTBI. **(A)** Alpha-tocopherol levels in serum for total patients without (n = 80) versus with a history of mTBI (n = 87), male patients without (n = 41) versus with history of mTBI (n = 61), and female patients without (n = 39) versus with a history of mTBI (n = 26). **(B)** Coenzyme-q10 levels in serum for total patients without (n = 80) versus with history of mTBI (n = 87), male patients without (n = 41) versus with history of mTBI (n = 61), and female patients without (n = 39) versus with history of mTBI (n = 26). **(C)** Cysteine levels in serum for total patients without (n = 37) versus with history of mTBI (n = 48), male patients without (n = 16) versus with history of mTBI (n = 30), and female patients without (n = 21) versus with history of mTBI (n = 18). **(D)** Selenium levels in serum for total patients without (n = 37) versus with history of mTBI (n = 48), male patients without (n = 16) versus with history of mTBI (n = 30), and female patients without (n = 21) versus with history of mTBI (n = 18). Boxplots represent the minimum, maximum, median, mean (diamond symbol), first/third quantile, and outlying values (≤/≥ lower/upper hinge + 1.5 * interquartile range). Statistical differences are analyzed by the Mann Whitney U-Test. # indicates trending $p < 0.10$, * indicates $p < 0.05$, and ** indicates $p < 0.01$.

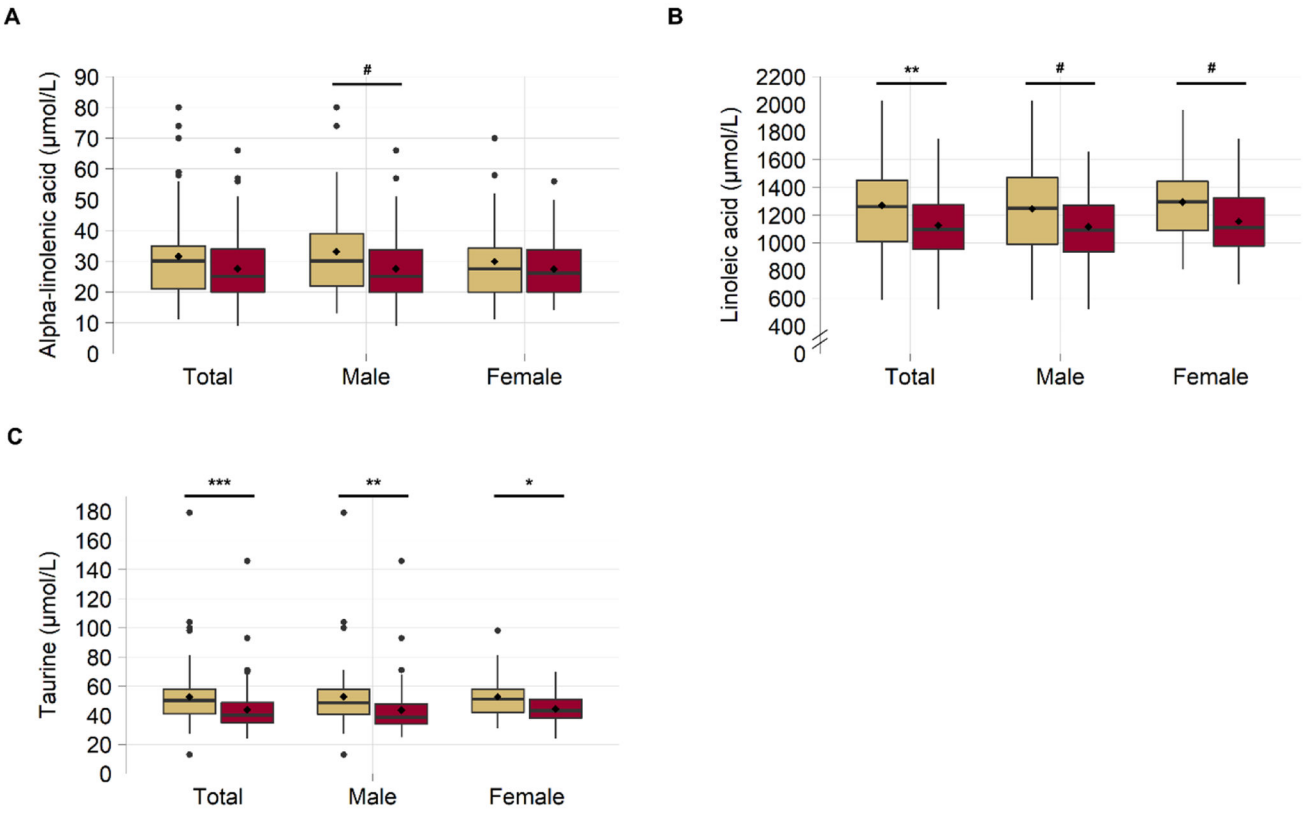

Fig. 2. Significantly or trending significantly reduced levels of plasma antioxidant biomarkers in total, male, and female patients with versus without a history of mTBI.

In each graph, the gold plot represents populations without a history of mTBI and the crimson plot represents populations with a history of mTBI. **(A)** Alpha-linolenic levels in plasma for total patients without (n = 81) versus with history of mTBI (n = 88), male patients without (n = 41) versus with history of mTBI (n = 62), and female patients without (n = 40) versus with history of mTBI (n = 26). **(B)** Linoleic acid levels in plasma for total patients without (n = 81) versus with history of mTBI (n = 88), male patients without (n = 41) versus with history of mTBI (n = 62), and female patients without (n = 40) versus with history of mTBI (n = 26). **(C)** Taurine levels in plasma for total patients without (n = 77) versus with history of mTBI (n = 86), male patients without (n = 40) versus with history of mTBI (n = 62), and female patients without (n = 37) versus with history of mTBI (n = 24). Boxplots represent the minimum, maximum, median, mean (diamond symbol), first/third quantile, and outlying values (≤/≥ lower/upper hinge + 1.5 * interquartile range). Statistical differences are analyzed by the Mann Whitney U-Test. # indicates trending $p <$ 0.10, * indicates $p < 0.05$, ** indicates $p < 0.01$, and *** indicates $p < 0.001$.

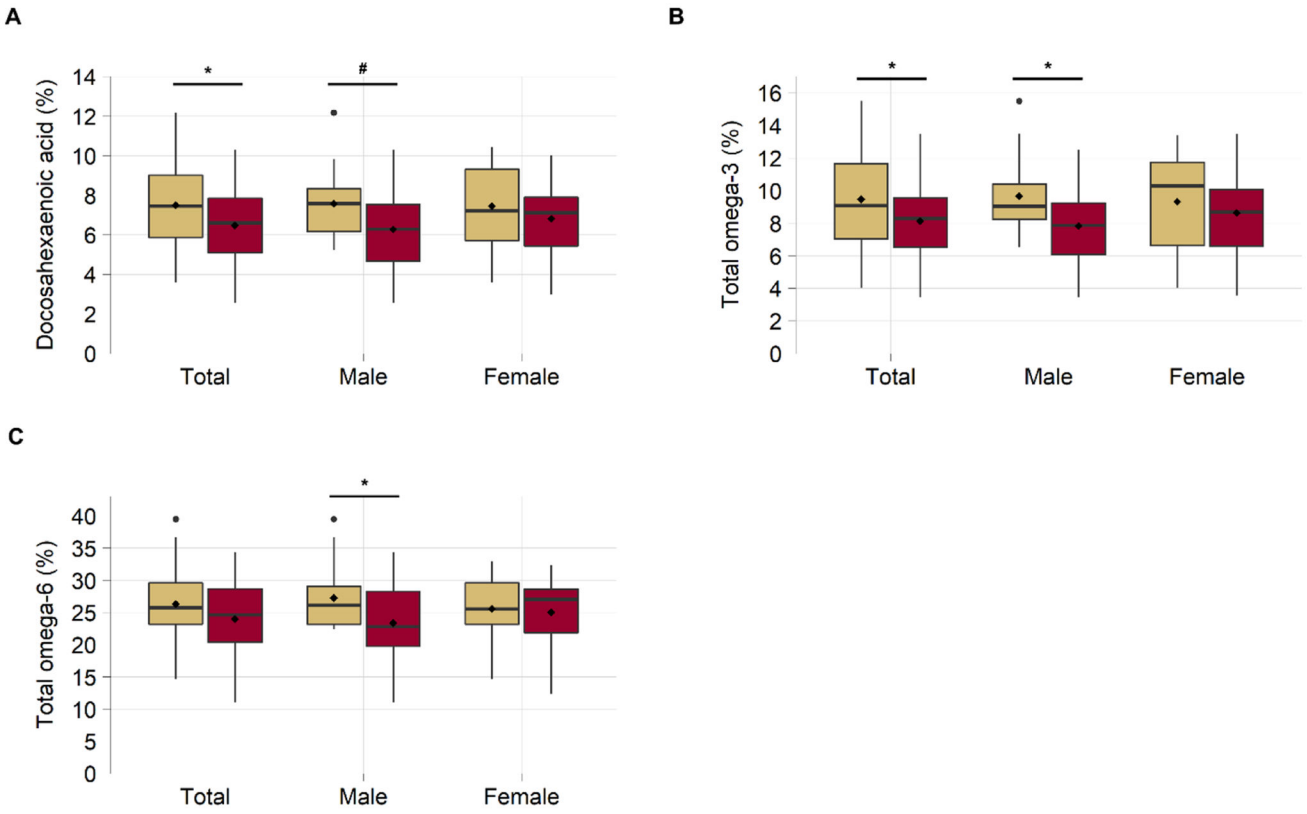

Fig. 3. Significantly or trending significantly reduced levels of RBC antioxidant biomarkers in total, male, and female patients with versus without a history of mTBI.

In each graph, the gold plot represents populations without a history of mTBI and the crimson plot represents populations with a history of mTBI. **(A)** Docosahexaenoic acid levels in RBCs for total patients without (n = 37) versus with history of mTBI (n = 48), male patients without (n = 16) versus with history of mTBI (n = 30), and female patients without (n = 21) versus with history of mTBI (n = 18). **(B)** Total omega-3 levels in RBCs for total patients without (n = 37) versus with history of mTBI (n = 48), male patients without (n = 16) versus with history of mTBI (n = 30), and female patients without (n = 21) versus with history of mTBI (n = 18). **(C)** Total omega-6 levels in RBCs for total patients without (n = 37) versus with history of mTBI (n = 48), male patients without (n = 16) versus with history of mTBI (n = 30), and female patients without (n = 21) versus with history of mTBI (n = 18). Boxplots represent the minimum, maximum, median, mean (diamond symbol), first/third quantile, and outlying values (≤/≥ lower/upper hinge + 1.5 * interquartile range). Statistical differences are analyzed by the Mann Whitney U-Test. # indicates trending p < 0.10 and * indicates p < 0.05.

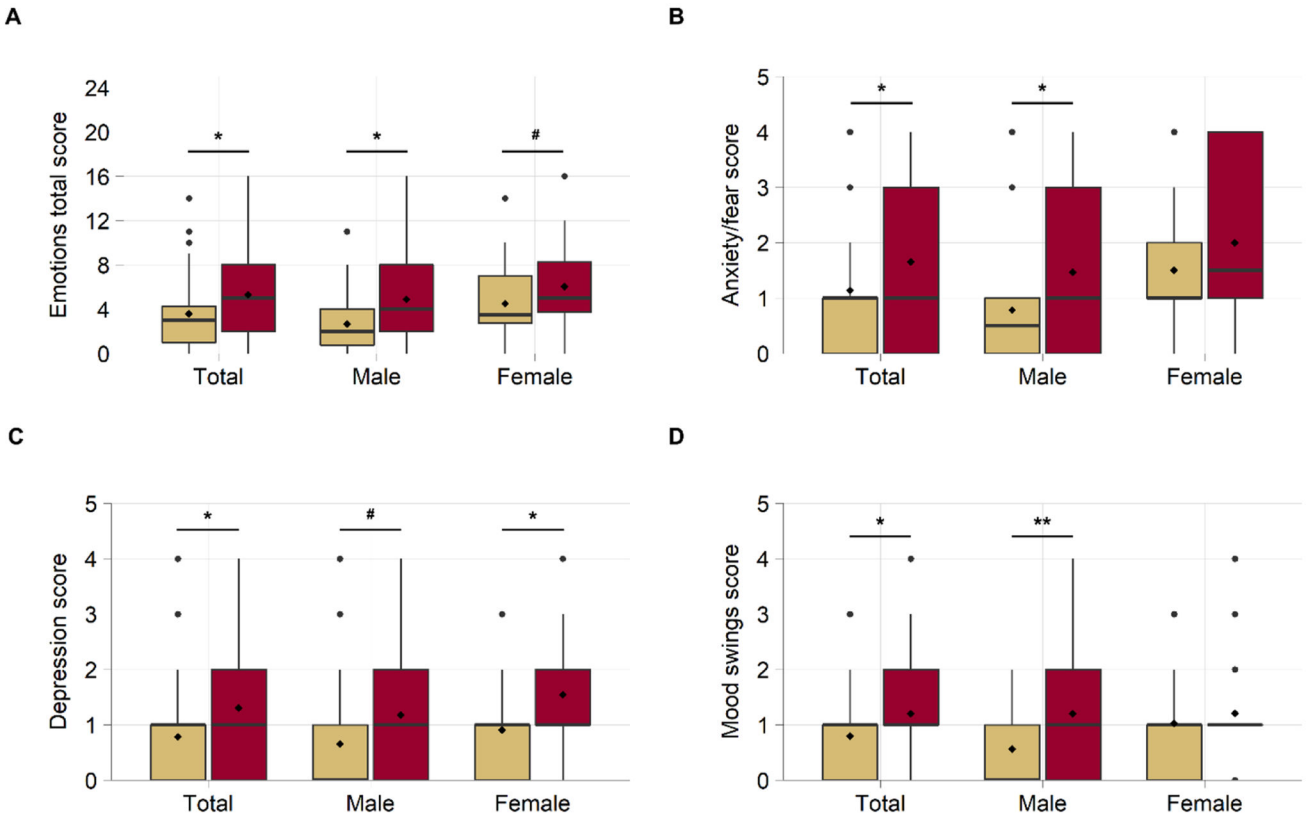

Fig. 4. Significantly or trending significantly worsened symptoms pertaining to emotional state in total, male, and female patients with versus without a history of mTBI.

In each graph, the gold plot represents populations without a history of mTBI and the crimson plot represents populations with a history of mTBI. **(A)** Emotion symptom category total score for total patients without ($n = 64$) versus with history of mTBI ($n = 69$), male patients without ($n = 32$) versus with history of mTBI ($n = 45$), and female patients without ($n = 32$) versus with history of mTBI ($n = 24$). **(B)** Anxiety/fear symptom score for total patients without ($n = 64$) versus with history of mTBI ($n = 69$), male patients without ($n = 32$) versus with history of mTBI ($n = 45$), and female patients without ($n = 32$) versus with history of mTBI ($n = 24$). **(C)** Depression symptom score for total patients without ($n = 64$) versus with history of mTBI ($n = 69$), male patients without ($n = 32$) versus with history of mTBI ($n = 45$), and female patients without ($n = 32$) versus with history of mTBI ($n = 24$). **(D)** Mood swing symptom score for total patients without ($n = 64$) versus with history of mTBI ($n = 69$), male patients without ($n = 32$) versus with history of mTBI ($n = 45$), and female patients without ($n = 32$) versus with history of mTBI ($n = 24$). Boxplots represent the minimum, maximum, median, mean (diamond symbol), first/third quantile, and outlying values (≤/≥ lower/upper hinge + 1.5 * interquartile range). Statistical differences are analyzed by the Mann Whitney U-Test. # indicates trending $p < 0.10$, * indicates $p < 0.05$, and ** indicates $p < 0.01$.

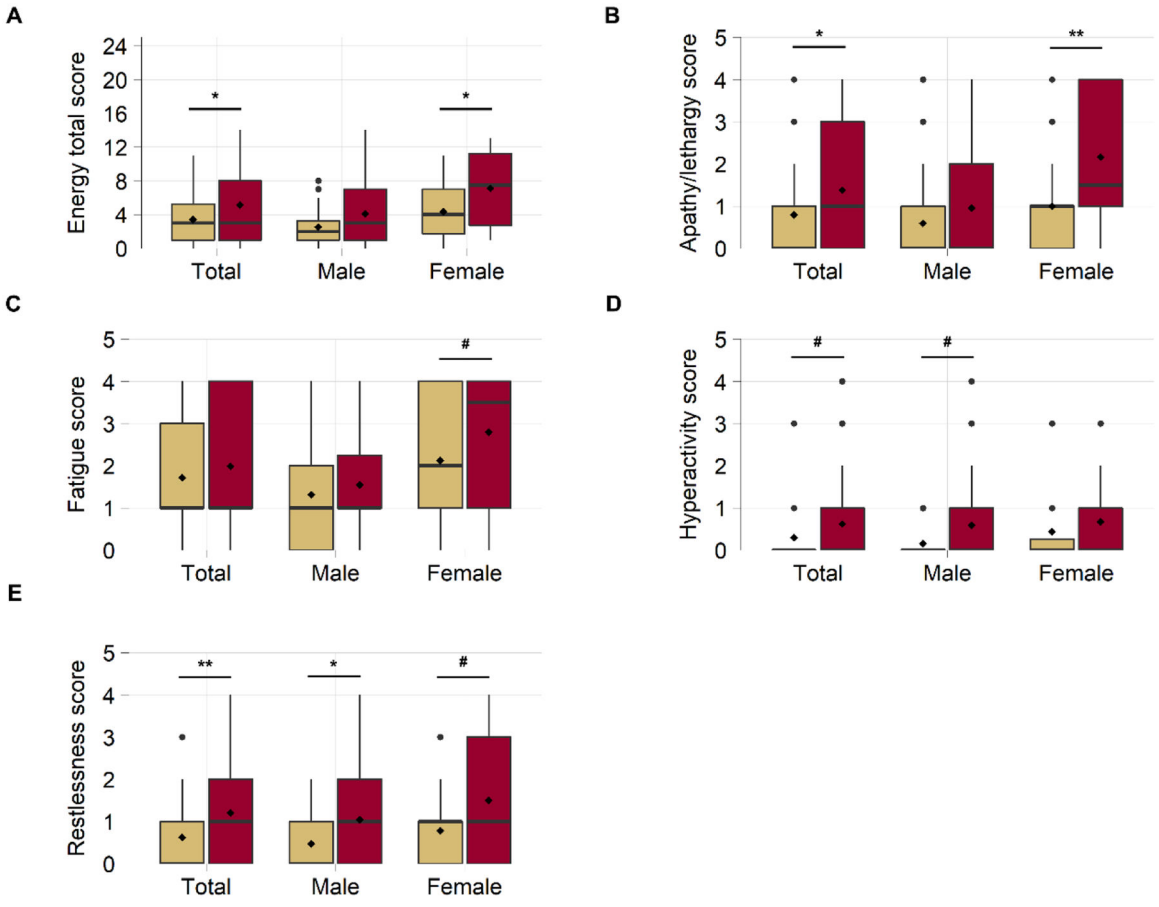

Fig. 5. Significantly or trending significantly worsened symptoms pertaining to energy in total, male, and female patients with versus without a history of mTBI.

In each graph, the gold plot represents populations without a history of mTBI and the crimson plot represents populations with a history of mTBI. **(A)** Energy symptom category total score for total patients without (n = 64) versus with history of mTBI (n = 69), male patients without (n = 32) versus with history of mTBI (n = 45), and female patients without (n = 32) versus with history of mTBI (n = 24). **(B)** Apathy/lethargy symptom score for total patients without (n = 64) versus with history of mTBI (n = 69), male patients without (n = 32) versus with history of mTBI (n = 45), and female patients without (n = 32) versus with history of mTBI (n = 24). **(C)** Fatigue symptom score for total patients without (n = 64) versus with history of mTBI (n = 68), male patients without (n = 32) versus with history of mTBI (n = 44), and female patients without (n = 32) versus with history of mTBI (n = 24). **(D)** Hyperactivity symptom score for total patients without (n = 64) versus with history of mTBI (n = 68), male patients without (n = 32) versus with history of mTBI (n = 44), and female patients without (n = 32) versus with history of mTBI (n = 24). **(E)** Restlessness symptom score for total patients without (n = 64) versus with history of mTBI (n = 69), male patients without (n = 32) versus with history of mTBI (n = 45), and female patients without (n = 32) versus with history of mTBI (n = 24). Boxplots represent the minimum, maximum, median, mean (diamond symbol), first/third quantile, and outlying values (≤/≥ lower/upper

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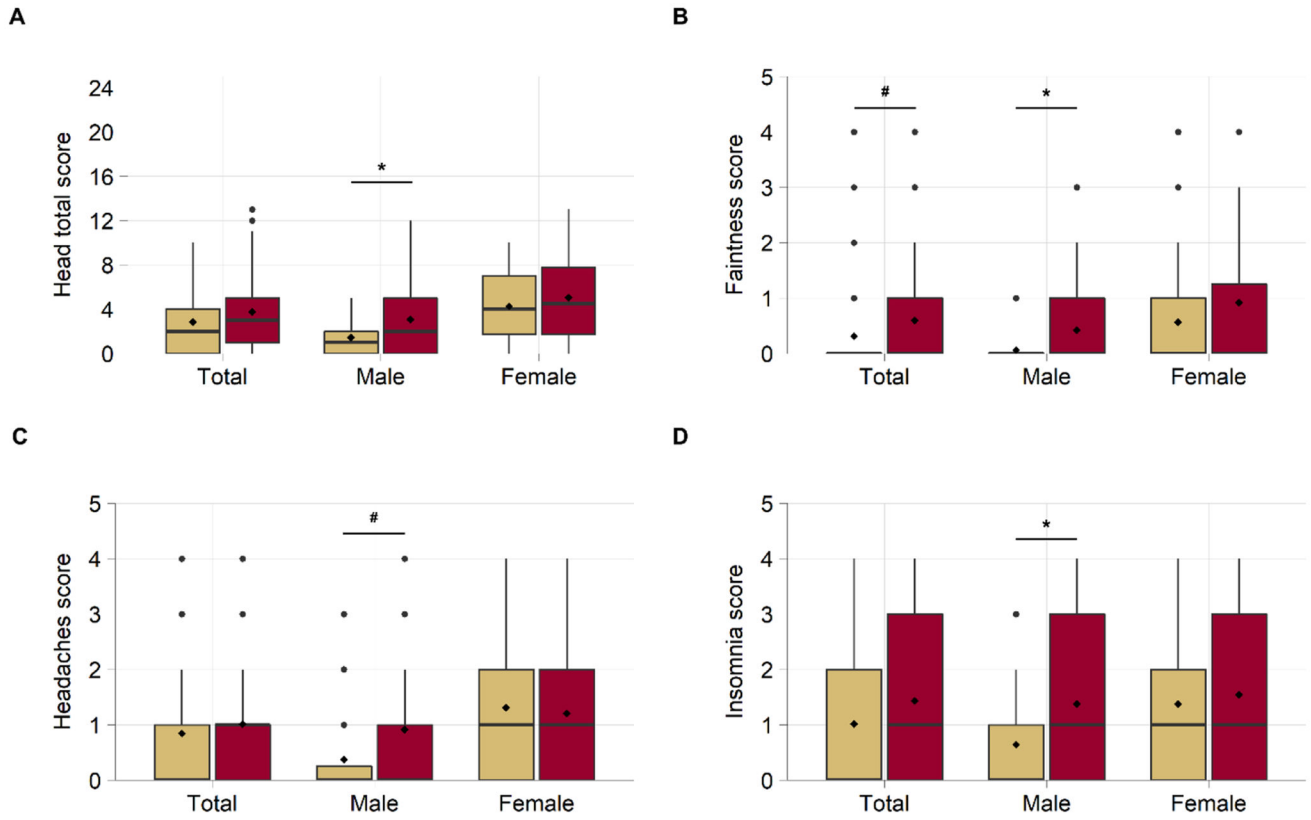

Fig. 6. Significantly or trending significantly worsened head symptoms in total, male, and female patients with versus without a history of mTBI.

In each graph, the gold plot represents populations without a history of mTBI and the crimson plot represents populations with a history of mTBI. **(A)** Head symptom total score for total patients without (n = 64) versus with history of mTBI (n = 69), male patients without (n = 32) versus with history of mTBI (n = 45), and female patients without (n = 32) versus with history of mTBI (n = 24). **(B)** Faintness symptom score for total patients without (n = 64) versus with history of mTBI (n = 69), male patients without (n = 32) versus with history of mTBI (n = 45), and female patients without (n = 32) versus female patients with history of mTBI (n = 24). **(C)** Headache symptom score for total patients without (n = 64) versus with history of mTBI (n = 69), male patients without (n = 32) versus with history of mTBI (n = 45), and female patients without (n = 32) versus with a history of mTBI (n = 24). **(D)** Insomnia symptom score for total patients without (n = 63) versus with history of mTBI (n = 69), male patients without (n = 31) versus with a history of mTBI (n = 45), and female patients without (n = 32) versus with a history of mTBI (n = 24). Boxplots represent the minimum, maximum, median, mean (diamond symbol), first/third quantile, and outlying values (≤/≥ lower/upper hinge + 1.5 * interquartile range). Statistical differences are analyzed by the Mann Whitney U-Test. # indicates trending $p < 0.10$ and * indicates $p < 0.05$.

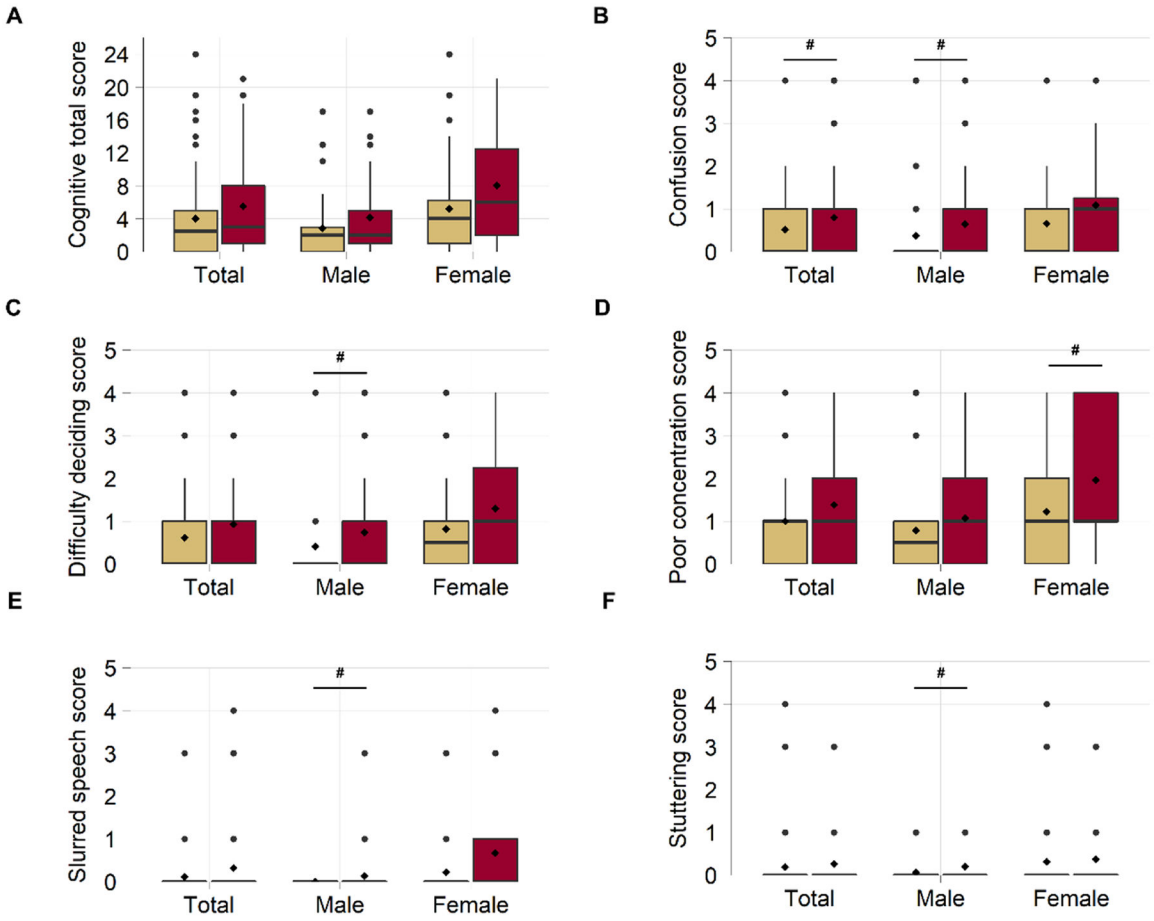

Fig. 7. Trending significantly worsened symptoms pertaining to cognition in total, male, and female patients with versus without a history of mTBI.

In each graph, the gold plot represents populations without a history of mTBI and the crimson plot represents populations with a history of mTBI. **(A)** Cognitive symptom total score for total patients without (n = 64) versus with history of mTBI (n = 69), male patients without (n = 32) versus with history of mTBI (n = 45), and female patients without (n = 32) versus female patients with history of mTBI (n = 24). **(B)** Confusion symptom score for total patients without (n = 64) versus with history of mTBI (n = 69), male patients without (n = 32) versus with history of mTBI (n = 45), and female patients without (n = 32) versus with history of mTBI (n = 24). **(C)** Difficulty deciding symptom score for total patients without (n = 64) versus with history of mTBI (n = 69), male patients without (n = 32) versus with history of mTBI (n = 45), and female patients without (n = 32) versus with history of mTBI (n = 24). **(D)** Poor concentration symptom score for total patients without (n = 64) versus with history of mTBI (n = 69), male patients without (n = 32) versus with history of mTBI (n = 45), and female patients without (n = 32) versus with history of mTBI (n = 24). **(E)** Slurred speech symptom score for total patients without (n = 64) versus with history of mTBI (n = 69), male patients without (n = 32) versus with history of mTBI (n = 45), and female patients without (n = 32) versus with history of mTBI (n = 24). **(F)** Stuttering symptom score for total patients without (n = 64) versus with history of mTBI (n = 69), male patients without (n = 32) versus with history of mTBI (n = 45), and female patients without

(n = 32) versus with history of mTBI (n = 24). Boxplots represent the minimum, maximum, median, mean (diamond symbol), first/third quantile, and outlying values (≤/≥ lower/upper hinge + 1.5 * interquartile range). Statistical differences are analyzed by the Mann Whitney U-Test. # indicates trending $p < 0.10$.

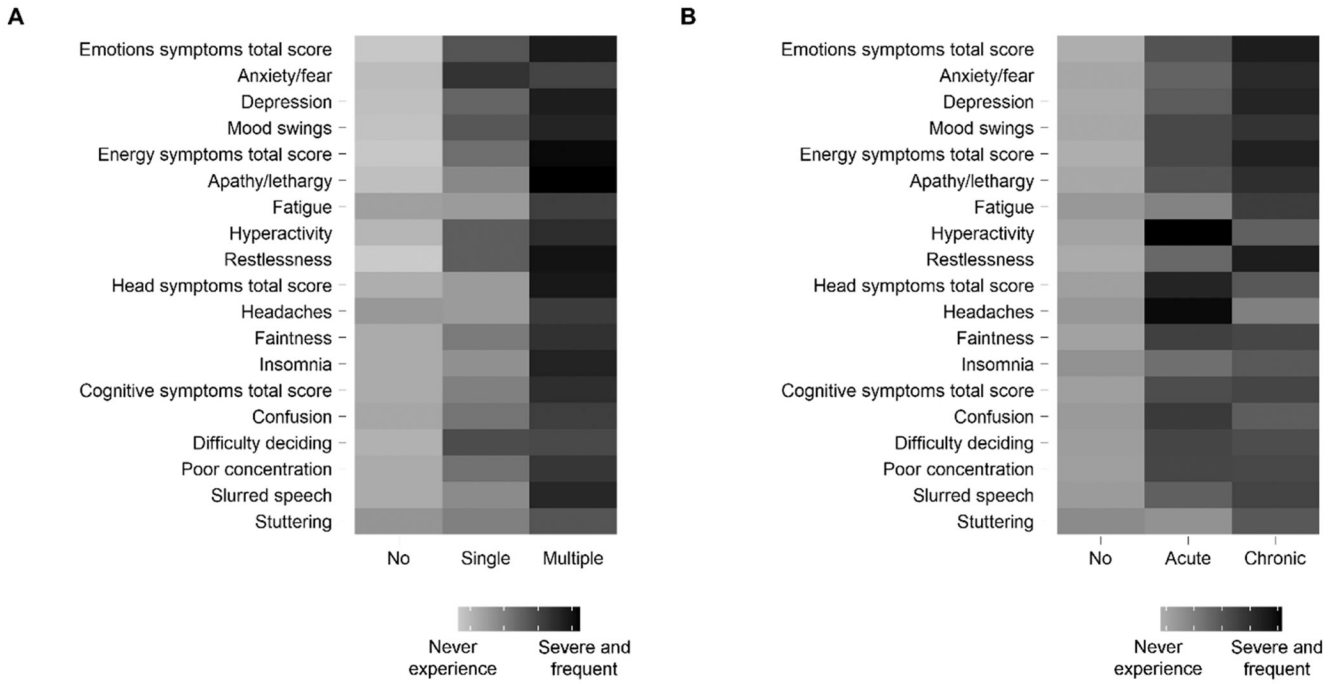

Fig. 8. Qualitative differences in emotional, energy, head, and cognitive symptom severity and frequency between patients with no history of mTBI versus patients with a past single mTBI or with a history of multiple mTBIs and between patients with no history of mTBI versus patients with acute-subchronic mTBI or chronic mTBI.

Total and individual symptom scores between **(A)** patients with no history of mTBI (No; $n = 82$), single mTBI (Single; $n = 27$), and multiple mTBI (Multiple; $n = 40$) or between **(B)** patients with no history of mTBI (No; $n = 82$), acute-subchronic mTBI (Acute; $n = 14$), and chronic mTBI (Chronic; $n = 32$) are displayed in a heatmap for qualitative comparison of symptom severity between these groups. Acute-subchronic mTBI patients presented to the clinic ≤ 1 year and chronic mTBI patients presented > 1 year since their most recent mTBI. A lighter shade indicates a lower symptom score, lessened severity, and less frequent, while a darker shade indicates a higher symptom score, worsened severity, and increased frequency.

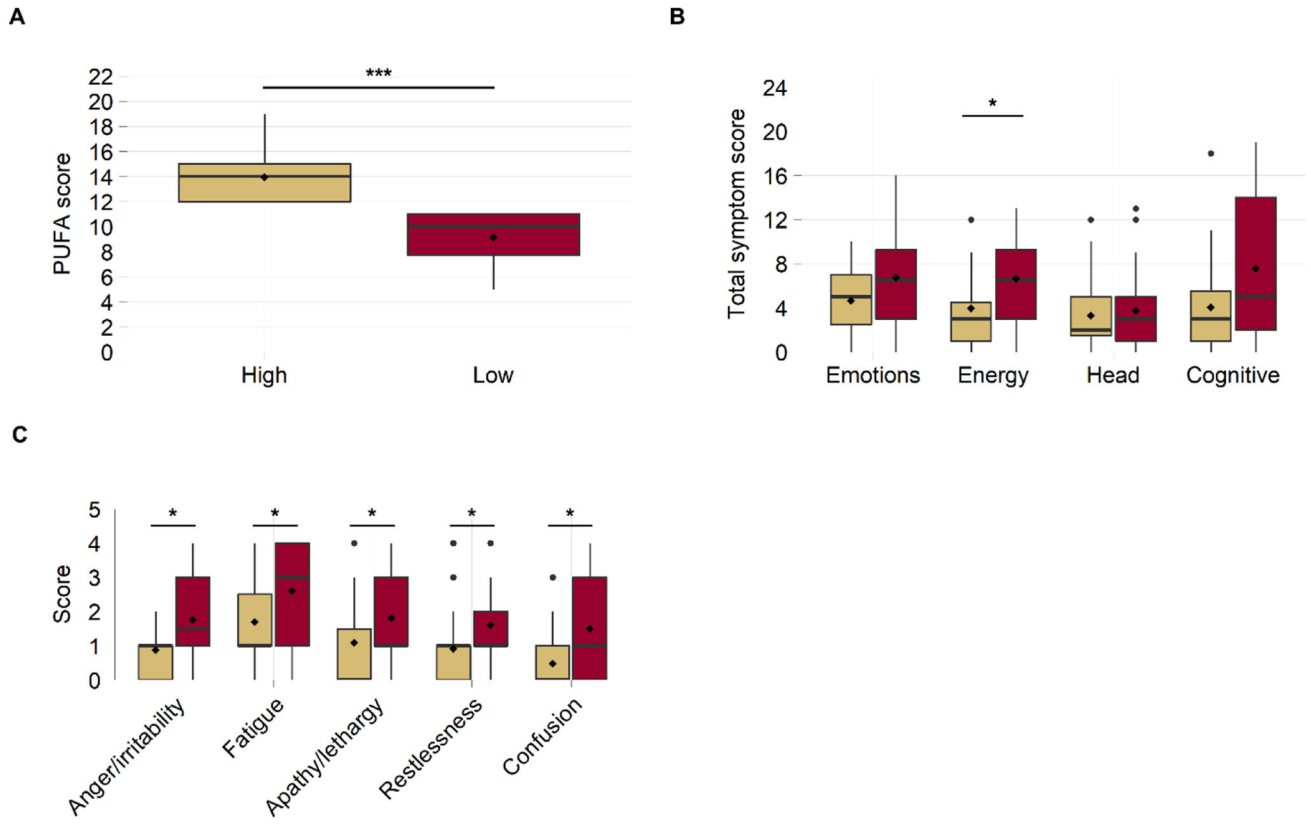

Fig. 9. Significantly worsened symptoms pertaining to emotional state, energy, head symptoms, or cognition in total history of mTBI patients with PUFA biomarker depletion when compared to history of mTBI patients with higher levels of PUFA biomarkers.

In each graph, the gold plot represents history of mTBI populations with a high PUFA biomarker score ($n = 23$) and the crimson bar represents populations with a low PUFA biomarker score ($n = 20$). **(A)** Polyunsaturated fatty acid biomarker scores were significantly different for total history of mTBI patients with a high versus low PUFA biomarker score. **(B)** MSQ symptom total scores analyzed in history of mTBI patients with a high versus low PUFA score. **(C)** Significantly worsened symptoms analyzed in history of mTBI patients with a high versus low PUFA biomarker score. Boxplots represent the minimum, maximum, median, mean (diamond symbol), first/third quantile, and outlying values (≤/≥ lower/upper hinge + 1.5 * interquartile range). The Mann Whitney U-Test was used to analyze statistical differences in PUFA scores and symptom scores. * indicates $p < 0.05$ and *** indicates $p < 0.001$.

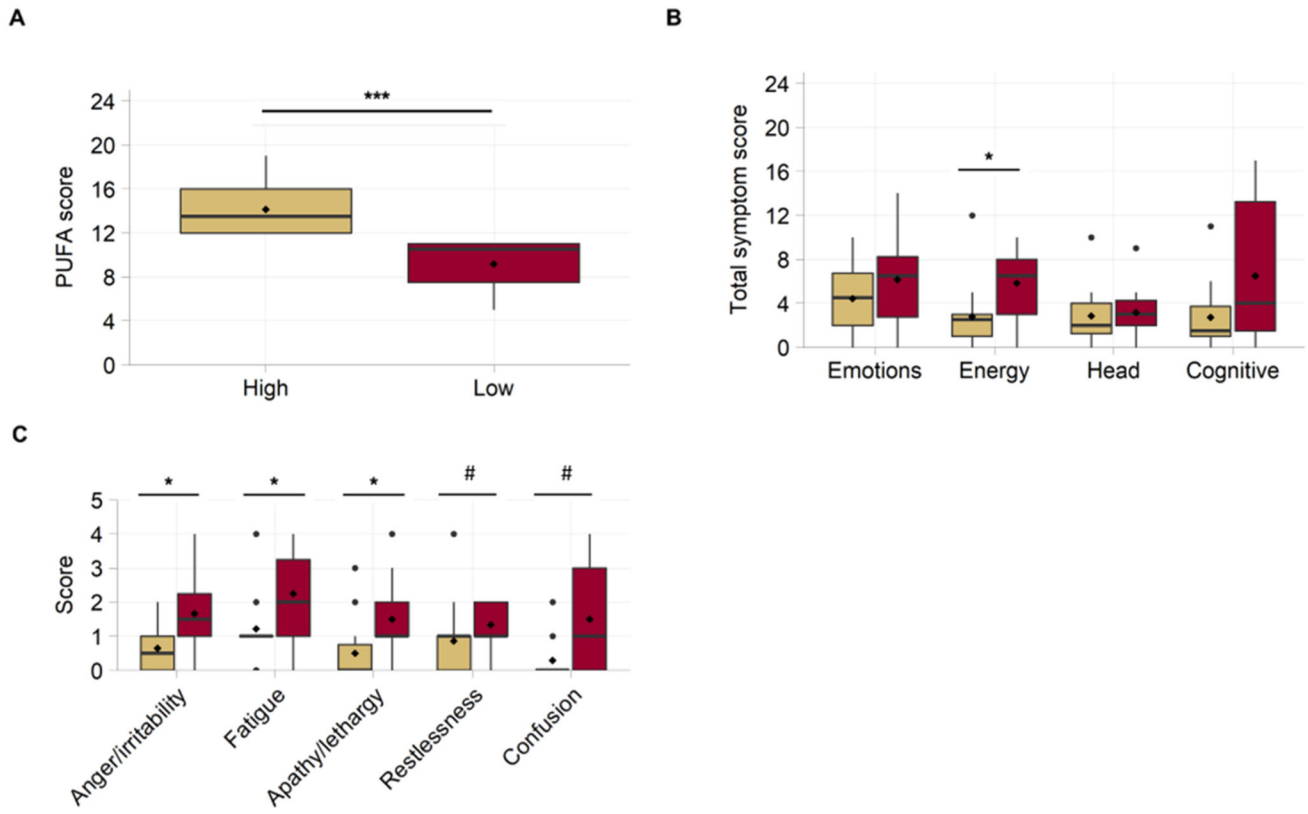

Fig. 10. Significantly worsened symptoms pertaining to emotional state, energy, head symptoms, or cognition in male history of mTBI patients with PUFA biomarker depletion when compared to male history of mTBI patients with higher levels of PUFA biomarkers.

In each graph, the gold plot represents male history of mTBI populations with a high PUFA biomarker score ($n = 14$) and the crimson bar represents male populations with a low PUFA biomarker score ($n = 12$). **(A)** Polyunsaturated fatty acid biomarker scores were significantly different for male history of mTBI patients with a high versus low PUFA biomarker score. **(B)** MSQ symptom total scores analyzed in history of male mTBI patients with a high versus low PUFA score. **(C)** Significantly worsened symptoms analyzed in male history of mTBI patients with a high versus low PUFA biomarker score. Boxplots represent the minimum, maximum, median, mean (diamond symbol), first/third quantile, and outlying values (≤/≥ lower/upper hinge + 1.5 * interquartile range). The Mann Whitney U-Test was used to analyze statistical differences in PUFA scores and symptom scores. * indicates $p < 0.05$ and *** indicates $p < 0.001$. # indicates trending $p < 0.10$.

Table 1

Age and sex of patients without and with a history of mTBI.

Age is displayed for total (n = 170), male, and female patients without versus with a history of mTBI. Age is reported in years as mean ± SEM (range). The Mann Whitney U-Test was used to analyze continuous data and Pearson Chi Square Test was used to analyze categorical data differences between groups. Significant p values ($p < 0.05$) are displayed as bolded text. Z statistics and effect sizes (r) are also displayed for significant continuous data.

	Patients without history of mTBI (n = 82)	Patients with history of mTBI (n = 88)	P-value	Z statistic	Effect size (r)
Age	46.16 ± 1.85 (17 – 83)	38.95 ± 1.81 (16 – 80)	**0.003**	2.957	0.226
Males	n= 42	n= 62	**0.010**		
Females	n= 40	n= 26			
Male age	48.62 ± 2.66 (24 – 83)	37.55 ± 2.09 (16 – 80)	**0.001**	3.361	0.330
Female age	43.58 ± 2.55 (17 – 77)	42.31 ± 3.50 (19 – 78)	0.694		

Table 2

Age, sex, and cause of most recent mTBI for patients with a history of mTBI.

Age is displayed for mTBI patient populations. Acute-subchronic mTBI patients presented to the clinic ≤ 1 year and chronic mTBI patients presented to the clinic > 1 year since their most recent mTBI. Age is reported in years as mean ± SEM (range). The Mann Whitney U-Test was used to analyze continuous data and Pearson Chi Square Test was used to analyze categorical data differences between groups. Significant *p* values ($p < 0.05$) are displayed as bolded text. Z statistics and effect sizes (r) are also displayed for significant continuous data.

	Patients with history of mTBI (*n* = 88)	***p*-value**	**Z statistic**	**Effect size (r)**
Male age (*n* = 62)	37.55 ± 2.09 (16 – 80)	0.247		
Female age (*n* = 26)	42.31 ± 3.50 (19 – 78)			
Cause of most recent mTBI (Both sexes; *n* = 88) (Not reported; *n* = 42)	Sport: *n* = 31 Fall: *n* = 9 Car accident: *n* = 3 Blunt injury: *n* = 3			
Males (*n* = 62) (Not reported; *n* = 33)	Sport: *n* = 27 Fall: *n* = 1 Car accident: *n* = 0 Blunt injury: *n* = 1	**<0.001**		
Females (n = 26) (Not reported; *n* = 9)	Sport: *n* = 4 Fall: *n* = 8 Car accident: *n* = 3 Blunt injury: *n* = 2			
Patients single mTBI	*n*= 27			
Age (Both sexes)	41.26 ± 3.20 (21 – 72)			
Male age (*n* = 18)	38.94 ± 3.98 (21 – 72)	0.275		
Female age (*n* = 9)	45.89 ± 5.32 (22 – 70)			
Patients with multiple mTBI	*n* = 40			
Age (Both sexes)	39.78 ± 2.84 (16 – 79)			
Male age (*n* = 25)	38.60 ± 3.43 (16 – 79)	0.699		
Female age (*n* = 15)	41.73 ± 5.06 (19 – 78)			
Patients with acute-subchronic mTBI	*n* = 14			
Age (Both sexes)	30.21 ± 3.97 (19 – 78)			
Male age (*n* = 7)	26.00 ± 1.54 (20 – 31)	0.805		
Female age (*n* = 7)	34.43 ± 7.75 (19 – 78)			
Patients with chronic mTBI	*n* = 32			
Age (Both sexes)	42.16 ± 2.75 (21 – 70)			
Male age (*n* = 18)	36.11 ± 3.30 (21 – 67)	**0.009**	0.320	0.086
Female age (*n* = 14)	49.93 ± 3.81 (24 – 70)			