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RESEARCH ARTICLE

Insights Into Long COVID Fatigue Biology: Potential Treatment Implications Using Multi-Targeted Action of AXA1125, a Novel Endogenous Metabolic Modulator Composition

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ABSTRACT

With ongoing global research efforts to tackle coronavirus disease 2019 (COVID-19), increasing attention is directed toward the long-term sequelae of COVID, entitled "long COVID" or post-acute sequelae of COVID-19. These long-COVID symptoms persist beyond 12 weeks in over 10%–40% of patients, with exertional fatigue predominant in at least 50%. Scientific evidence has linked long COVID fatigue with mitochondrial dysfunction and energetic dysregulation in multiple biological pathways. Single target-directed treatments could be insufficient to treat these heterogeneous disorders. A novel multi-targeted therapeutic strategy could better address long COVID fatigue by restoring mitochondrial function. Our systems biology platform identified mechanisms implicated in long COVID and prioritized the composition of endogenous metabolic modulators focused on amino acid combinations, related precursors, and metabolites with the potential to address mitochondrial dysfunction. AXA1125 is a novel composition of five amino acids (Leucine, Isoleucine, Valine, Arginine, and Glutamine) and an amino acid derivative (N-acetylcysteine) that could safely target multifactorial disease pathophysiology of fatigue-dominant long COVID. Our phase IIa, double-blind, randomized trial (NCT05152849) in exertional fatigue patients associated with long COVID interrogated this proposition and showed promising results. We hypothesize that AXA1125 holds the potential for improving functional clinical outcomes by targeting multiple disease pathways and improving mitochondrial function and energetics.

Keywords: amino acid, energetic dysregulation, exercise tolerance, fatigue, long COVID, mitochondrial dysfunction, systems biology.

Introduction

Globally, over 500 million people have been infected with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)[1]. Emerging clinical evidence indicates that some patients experience long-term symptoms irrespective of the disease severity. The reported frequency of these long-term symptoms ranges from 10% to 30%[2-5]. Symptoms may persist from months to years after the initial infection[6-8], and understanding of the underlying mechanisms driving the longevity and heterogeneity of symptoms is evolving. First referred to as post-acute sequelae of SARS-CoV-2 infection by the National Institutes of Health[9], these persistent symptoms are also referred to as "long COVID" or post-acute COVID-19 syndrome[10], and have a long-lasting impact on the affected patients and caregivers.

The latest pooled estimates from over 41 studies show that the regional prevalence of long COVID was lower in the United States when compared with Asia and Europe, with prevalence ranging from ~30% to 50%, based on the definititions reported in this meta-analysis[11]. Evidence from the Office of National Statistics in the United Kingdom indicates that over 2 million individuals in the region (3.1% of the United Kingdom population) had self-reported long COVID as of May 1, 2022[12]. Assessing the prevalence of long COVID is challenging considering factors including, but not limited to, variations in population, gender, geography, healthcare access, inconsistent use of long COVID case definitions, methodological quality, the nature of reporting (self-reporting or clinical diagnosis or survey questionnaire), the severity of acute phase, and time since infection[13]. Nevertheless, long COVID's impact beyond a post-epidemic syndrome is associated with poor quality of life, impacting physical and mental well-being[14,15]. This impact unequivocally supports the critical need to develop therapeutic strategies to combat this syndrome, where currently, there is no approved treatment.

Despite extensive ongoing research and investigations, the pathophysiology of long COVID remains complex and evolving[16,17]. The multifactorial and heterogenous nature of long COVID highlights the need to identify and understand its underlying biology, natural history, and symptoms defining disease progression. Moreover, there is a requirement to explore the potential persistent hyperinflammatory state, the impact of the immune system, organ damage, viral activity, and predisposing factors for developing the syndrome[18-21].

The clinical symptoms of long COVID are usually varied and affect multiple organ systems, including the neurological, cardiovascular, musculoskeletal, respiratory, dermatological, endocrine, and gastrointestinal systems[5]. These symptoms adversely affect the day-to-day activities and quality of life of affected patients. Of the protracted and fluctuating symptoms associated with long COVID, fatigue remains one of the most consistent, persistent, and debilitating aspects of this complex syndrome[11,22-30]. A systematic review of 25 studies (N=9,751) reported that at least 50% of individuals with long COVID experienced fatigue[31]. Neuropsychologic symptoms, often referred to as "brain fog," follow in the frequency of occurrence[23,30-34]. The most extensive study of

long-term health effects in COVID-19 patients to date showed that while long COVID prevalence was associated with acute disease severity, a sizeable portion of individuals with the milder disease is also impacted (hospitalized [49.98%], symptomatic [27.48%] and asymptomatic [18.95%] patients)[35]. Emerging evidence from the ongoing investigations into the mechanisms and determinants of such persistent symptoms implicates mitochondrial dysregulation and inflammatory pathways in exertional fatigue due to long COVID[36-38].

The biological cascade of SARS-CoV-2 infection and disease progression to long COVID is driven by the dysregulation of multiple biological pathways, limiting the effectiveness of single-target therapies (Figure 1). Multifactorial disorders such as long COVID could benefit from a multi-targeted drug combination therapeutic approach[19,39], offering advancements in the novel, next-generation tools and technologies and systems biology research which can reveal underlying disease mechanisms and highlight viable therapeutic hypotheses[28,40-43]. Such approaches offer opportunities to advance multi-targeting network-based tactics to elucidate disease mechanisms and advance therapeutic combination strategies against these mechanisms, combining advanced and large-scale biological investigation with state-of-the-art data science approaches. Axcella Therapeutics (United States of America) has an established research foundation that can support the specific design of endogenous metabolic modulator (EMM) compositions for multifactorial biological pathways, which could be involved in diseases[44]. With a focus on amino acid (AA) combinations and related precursors and metabolites, these formulations can concomitantly have the potential to address multiple facets of chronic, multifactorial diseases in a safe and tolerable manner, as demonstrated by clinical and non-clinical data. For example, the effects of EMMs in specific combination contributed to the development of AXA1125 to target the multifactorial pathophysiology that has been found to positively impact individuals with nonalcoholic steatohepatitis (NASH)[44,45]. AXA1125 comprises five AAs (Leucine [L], Isoleucine [I], Valine [V], Arginine [R], and Glutamine [Q] and an AA derivative N-acetylcysteine [Nac]; LIVRQNac; referred to as AXA1125 in human studies)[46].

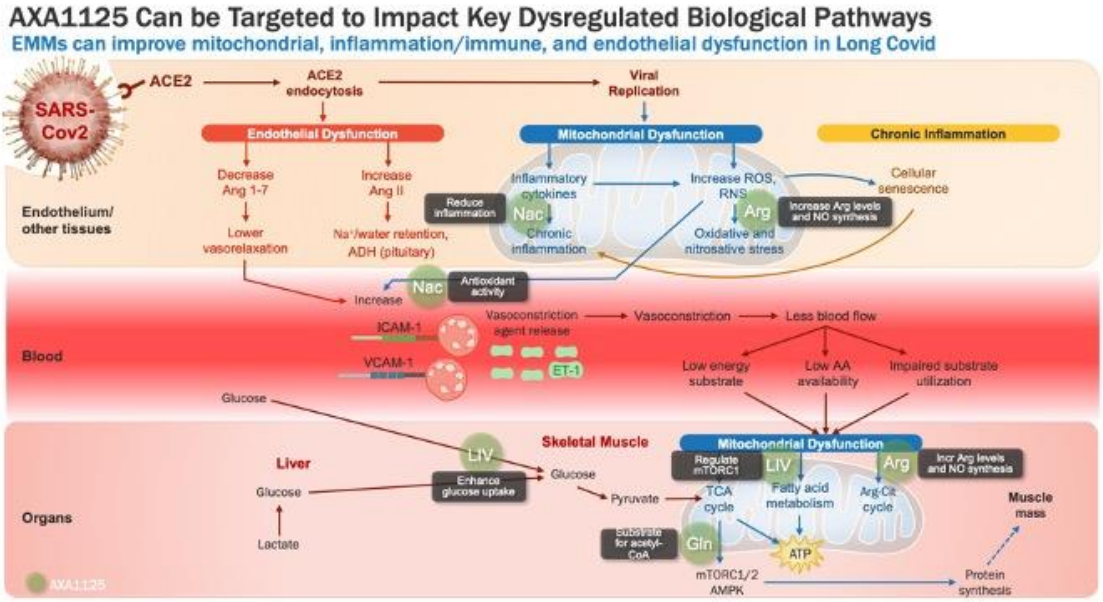

Figure 1: The key biological pathways that AXA1125 can target.
AXA1125 is a novel composition of five AAs (Leucine [L], Isoleucine [I], Valine [V], Arginine [R], and Glutamine [Q]) and an AA derivative (N-acetylcysteine), with multi-targeting potential to restore functioning of biological processes compromised in long COVID, namely, mitochondrial energetics, inflammation, oxidative stress, and endothelial function. Branched-chain AAs (i.e., L, I, V) play a crucial role in improving mitochondrial bioenergetics and reactive oxygen species (ROS) scavenging by modulating the mammalian target of the rapamycin complex/endothelial nitric oxide signaling pathway. Arginine is a crucial substrate for nitric oxide (NO) synthesis, and its metabolism plays multiple roles in vascular biology, mediated primarily through NO-dependent mechanisms. Glutamine, the most abundant AA in the body, plays an anaplerotic role by replenishing TCA cycle intermediates to generate reducing equivalents that drive the mitochondrial respiratory chain. Nac is an antioxidant that also plays a neuroprotective role, inhibits oxidative stress, reduces inflammation, and replenishes reduced antioxidant enzyme levels.

Here, we hypothesize that AXA1125, a novel EMM composition, has the potential to restore multiple aspects implicated in long COVID, including dysregulated energetics, metabolic hijacking, increased oxidative stress and inflammation, and restoring cellular preference for oxidative phosphorylation over glycolysis, thereby restoring cellular energy metabolism through its multi-targeted mechanism of action.

Evidence of mitochondrial dysfunction and inflammation in long COVID: An unmet need

Emerging non-clinical evidence suggests that SARS-CoV-2 infects cells and hijacks cellular metabolism to maximize viral production and replication, thereby dysregulating mitochondrial processes[47]. In patients with severe outcomes, mitochondrial disruption triggered by SARS-CoV-2 involves high ferritin levels, leading to oxidative stress and impaired mitochondrial function[48,49]. Moreover, persistent viral fragments can continue to exacerbate and suppress mitochondrial function, leading to sustained cellular stress[50]. Infection of endothelial cells with SARS-CoV-2 promotes mitochondrial dysfunction, vascular inflammation, increases mitochondrial deoxyribonucleic acid (DNA) release, and activates toll-like receptor 9 signaling, inducing inflammatory responses that could lead to cellular exhaustion and immune metabolic dysfunction[51]. This process may contribute to

a cytokine storm and thrombotic complications in patients with severe COVID-19 infection[52]. Consistent with these observations, our internal research knowledge database and extensive natural language processing driven data mining of literature and clinical data registries have supported mitochondrial dysfunction as one of the primary overarching mechanisms for long COVID-induced fatigue, with implications on dysregulated bioenergetics and lipid metabolism, impaired immune response, increased oxidative stress and proinflammatory state, and dysregulated endothelial function[37,38]. Although we believe that mitochondria dysregulation is a key factor for long COVID patients, a comprehensive review by Davis et al. highlights that there are other key related mechanisms to also consider, including immune dysregulation, microbioata disruption, blood clotting, and neurological signaling dysfunction[21].

Long COVID symptoms, especially fatigue, are characteristic of the potentially damaging and largely inexplicable post-viral syndrome labeled myalgic encephalomyelitis or chronic fatigue syndrome[21,36]. These symptoms have also been linked to long lasting reduction in serotonin levels driven by interferon-related inflammatory factors that lead to memory impairment. There has been studies that have shown serotonin reulgated key mitohcondria functions in neurons[53]. Mitochondrial dysfunction and oxidative stress are implicated as significant vulnerability risk factors in long COVID patients[54]. Mitochondria yield 90% to 95% of the body's total energy by producing adenosine triphosphate (ATP) through oxidative phosphorylation[55], while the rest of the body's energy is generated through glycolysis in the cytoplasm[56]. Mitochondria are also involved in redox signaling, glycemic regulation, and the cellular proinflammatory response[57,58]. Dysfunction in any of these pathways could lead to clinical symptoms of fatigue, muscle weakness, and cognitive decline[58-62].

Monocytes and macrophages play a central role in the pathogenicity of SARS-CoV-2[63]. These immune cells adapt their metabolism and become highly glycolytic upon infection, nurturing rapid SARS-CoV-2 replication[64]. The viral infection prompts enhanced mitochondrial reactive oxygen species (ROS) production, stabilizing hypoxia-inducible factor-1α (HIF-1α) and consequently promoting glycolysis. HIF-1α directly inhibits T-cell responses, reducing epithelial cell survival[64]. The pathophysiological alterations in mitochondria lead to reduced oxidative capacity and antioxidant defense. This effect is driven by increased ROS production, reduced oxidative phosphorylation levels, increased pro-apoptotic signaling pathways, and decreased or impaired ATP synthesis, causing mitochondrial dysfunction[65]. Increases in ROS have been linked to pathologic cascades wherein oxidative damage causes impaired lipid metabolism, increases protein degradation and DNA damage and potentially accelerates neurodegenerative processes[66,67].

Restoring mitochondrial function could improve exertional fatigue due to long COVID by restoring favorable bioenergetics, reducing inflammation and oxidative stress, and improving endothelial function[36,68]. Various potential therapies using mitochondria-targeted molecules are being pursued to treat metabolic syndromes, improve mitochondrial health[69-76], and restore perturbed mitochondrial

metabolic pathways and redox balance[77]. Several markers, such as lactate, creatine, pyruvate, and AAs, are being investigated for their role in integrated mitochondrial stress response. Of these markers, two growth factors, i.e., fibroblast growth factor-21 and growth differentiation factor 15, have been quantified to diagnose mitochondrial dysfunction, particularly attributable to acute COVID-19 infection[38,78]. AXA1125 could potentially improve mitochondrial function by restoring cellular respiration/energetics and enhancing cellular response under higher metabolic demand conditions, for example, during exertion.

Treatment of a complex disease — multi-targeted therapeutics for restoring biological homeostasis in complex, heterogeneous diseases

The identification of successful treatment opportunities for multifactorial diseases is challenging. Single-target treatment approaches have often been unsuccessful in fully addressing the needs of complex and heterogeneous disorders[79-81]. Long COVID is a highly heterogeneous disorder with likely multiple underlying pathophysiological mechanisms[16,17]. The heterogeneity may be due to various reasons, including differing degrees of immunologic and inflammatory injury due to acute infection, patients' underlying responses and risk factors, and the expected sequelae of post-critical illness[30]. The prevalence, severity of symptoms, and the likelihood of multiple mechanisms underlying long COVID make a multi-targeted therapeutic approach a pressing unmet medical need.

Endogenous metabolic modulators describe a broad spectrum of molecular families, including AAs, fatty acids, other lipids, bile acids, ketone bodies, hormones, and other molecules. As a result of their action on multiple disease nodes and metabolic pathways in a diseased condition, EMMs can potentially restore metabolic homeostasis in human diseases involving underlying metabolic dysregulation[44,81-85]. Amino acids are critical constituents to life-sustaining biochemical processes underlying cellular metabolism and energetics, including the tricarboxylic acid (TCA) cycle, where the oxidation of carbohydrates, proteins, and fats converges[86]. Moreover, AAs and related metabolites and precursors are the primary controllers of metabolic regulation and homeostasis of the body[87-89]. Being native to the body, EMMs are generally well tolerated and recognized as safe in therapeutic application[45,90]. Therefore, with the appropriate combination of these EMMs, targeted modification of complex diseases could be achieved.

Our translational research and systems biology foundation support the specialized design of EMM compositions with the potential to impact multifactorial diseases. The same is endorsed by our pre-clinical and clinical data for NASH/nonalcoholic fatty liver disease (NAFLD), type 2 diabetes, inflammation, and muscle-related frailty[44,45,91]. Our therapeutic approach results from curating decades of research on individual AAs and their combinations. These roles of AAs can be leveraged to develop treatments for diseases with complex biology using novel disease-specific therapeutic EMM compositions[44]. We linked AA biology to putative underlying long COVID disease mechanisms, biomarkers, and symptoms

through our internal tools and platform-based process. Network biology and data mining approaches inform on published and evolving mechanisms in the disease condition under investigation. These mechanisms are subsequently interrogated based on clinical disease registries from the patient population to expand our understanding and could potentially unveil specific mechanisms implicated in long COVID or other complex disorders to target therapeutically.

Compositions of EMMs anchored by AAs can be designed to safely target and modulate multiple targets and pathways simultaneously and therefore have great potential as tools for a multifactorial approach to treating complex, heterogeneous diseases.

Amino acid combinations targeting mitochondrial dysfunction: Bridging the gap

Metabolomics analysis revealed that the lipid and AA metabolism super pathways were the most enriched between patients hospitalized with mild and severe COVID-19 and healthy controls[92]. Data on AA metabolism supports continued redox imbalance, impaired bioenergetics, and abnormal immune responses in patients with long COVID[38].

As substrates, AAs have demonstrated multi-targeted roles in regulating metabolism. Branched-chain AAs (i.e., L, I, V) play a crucial role in improving mitochondrial biogenesis, cellular energy metabolism, and ROS scavenging by modulating the mechanistic target of the rapamycin complex/ endothelial nitric oxide signaling pathway[93,94]. They provide a significant source of cellular energy via acetyl coenzyme A and succinyl coenzyme A generation[95]. Arginine is involved in multiple metabolic processes, and its depletion could disrupt several cellular and organ functions[96] and induce T-cell or endothelial dysfunction[97]. Arginine is a crucial substrate for nitric oxide (NO) synthesis, and its metabolism plays multiple roles in vascular biology and diseases, mediated primarily through NO-dependent mechanisms[98,99]. NO attenuates the reduction of cytochrome C oxidase, facilitating the release of intracellular superoxide from the mitochondria and providing signals for vasodilation[100]. The vascular relaxation induced through NO signaling and the regulation of adhesion marker expression on the endothelial surface improves circulatory dynamics and redox balance and could potentially mitigate COVID-19 vasculopathy[97-99]. Glutamine, the most abundant AA in the body, plays an anaplerotic role by replenishing TCA cycle intermediates to generate reducing equivalents that drive the mitochondrial respiratory chain[101,102]. A decrease in von Willebrand Factor (vWF) polymerization by Nac has been described in preclinical models[103], and the vWF-ADAMTS13 axis has been implicated in long COVID exercise intolerance[104]. The AA derivative, Nac, also plays a neuroprotective role by restoring mitochondrial dysfunction, inhibiting oxidative stress, and replenishing the reduced antioxidant enzyme levels[105-108].

The potential benefits of AXA1125 in long COVID include improved mitochondrial bioenergetics, reduced inflammation and oxidative stress, and improved endothelial function. Induced substrate mobilization and increased NO signaling could enhance perfusion and vascular conduction, and

reduce vascular inflammation, protein breakdown, and muscle fatigue post-exercise. Increased preferential fatty acid oxidation relative to glycolysis restores viral infection-driven bioenergetic changes, whether in presence or absence of viral fragments, and AXA1125 has been shown to increase expression for fatty acid metabolism genes in primary human hepatocytes exposed to free fatty acids and tumor necrosis factor-α (Figure 2). The potential of AXA1125 in increasing fatty acid oxidation, its impact on lipid metabolism, and its effects on inflammation, insulin resistance, and fibrosis have been established in pre-clinical and clinical studies on multifactorial diseases such as NASH/ NAFLD[45,91]. Taken together, we hypothesize that AXA1125 could potentially target the multifactorial pathophysiology of exertional fatigue associated with long COVID.

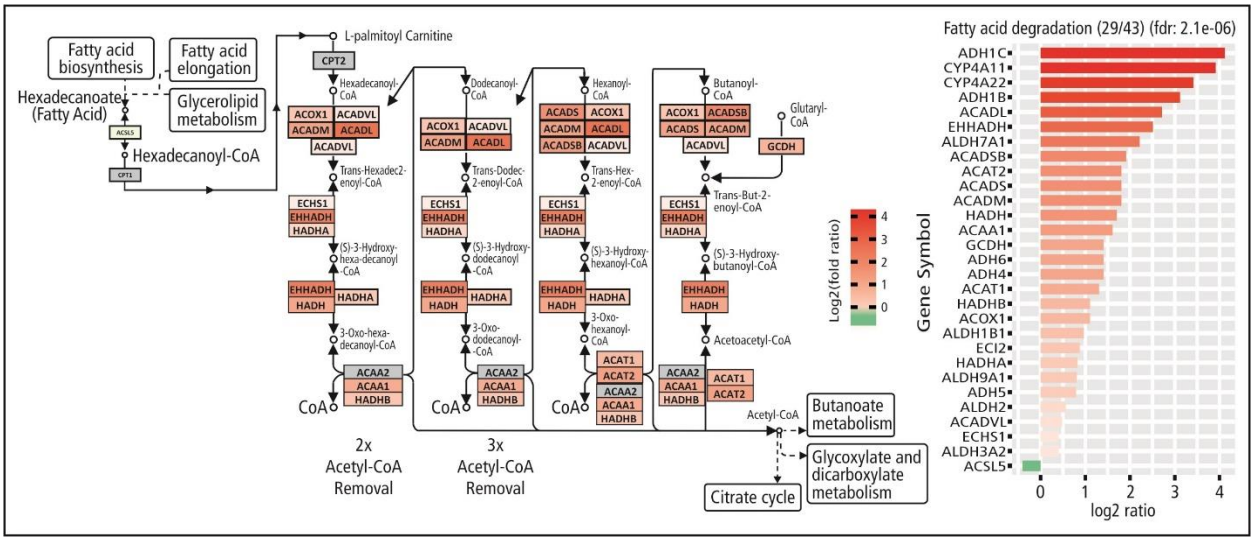

Figure 2: The effect of AXA1125 on the expression of genes involved in fatty acid metabolism in primary human hepatocytes exposed to free fatty acids and TNF-α(Journal of Hepatology, 77:S718–S718, 2022).

Synopsis of AXA1125 Clinical Proof-of-Concept Study

Currently, there are 12 ongoing trials for fatigue due to long COVID (https://www.clinicaltrials.gov). The translatability of our hypothesis was investigated in a single-center clinical trial (NCT05152849) conducted in collaboration with Oxford University Radcliffe (United Kingdom). This Phase IIa trial was a double-blind, randomized, placebo-controlled study evaluating the efficacy and safety of AXA1125 in patients with exertional fatigue associated with long COVID. This study was conducted according to the protocol, the ethical principles that have their origins in the Declaration of Helsinki, including the current International Council for Harmonization Good Clinical Practice Consolidated Guideline E6 R2, and all applicable national and local laws and regulations. Written and oral information about the study was provided to all participants in a language understandable by the participants. Written informed consent was obtained from each participant before any study procedures or assessments were performed. Each participant's willingness to participate in the study was documented in writing in a consent form signed and dated by the participant. Each investigator kept the

original consent forms, and copies were given to the participant. This study was approved by the Fast-Track Research Ethics Committee, Health Research Authority (Stratford, London, United Kingdom). In this trial, patients with fatigue-predominant long COVID were randomized (1:1) to receive AXA1125 (67.8 g/day) or a matched placebo in two doses for 28 days, with a one-week safety follow-up phase. The primary endpoint assessed the improvement in mitochondrial function within skeletal muscles from baseline to Day 28, evaluated through changes in the phosphocreatine recovery time, which was assessed using 31-phosphorus magnetic resonance spectroscopy and is a measure of oxidative capacity. An exploratory biomarker panel was utilized to evaluate the impact on overarching mechanisms implicated in mitochondrial dysfunction, including bioenergetics and lipid metabolism, oxidative stress and inflammation, endothelial function, and mitochondrial health. The key secondary endpoints in this trial included assessing lactate levels, a serum marker of mitochondrial dysfunction, and increased glycolysis[109-111], a 6-minute walk test, Chalder Fatigue Score, and safety and tolerability of AXA1125. Sixty participants were screened, and 41 were randomized and included in the final analysis. Study inclusion criteria were (i) 18–64 years of age, (ii) with clinically suspected COVID-19 ≥ 12 weeks before screening, (iii) displayed fatigue predominant long COVID, as defined by a total fatigue (bimodal) score of ≥8 on the Chalder Fatigue Questionnaire 11, and (iv) with a post-exertional skeletal muscle phosphocreatine recovery rate constant >50 s (a marker of impaired mitochondrial oxidative capacity) measured using 31-phosphorus magnetic resonance spectroscopy. Patients presenting with other possible causes of fatigue (e.g., heart failure, chronic cardiovascular, neurological, neuromuscular, or hepatic disease, hypothyroidism, or clinically significant anemia) were excluded. Intergroup comparisons for categorical endpoints were performed with the chi-square test. Continuous endpoints were analyzed using the analysis of covariance models with the change or percent change from baseline as the dependent variable and adjusted for baseline value. While the study did not show a statistically significant difference in the experimental biomarker primary endpoint due to a much higher than expected variability in this patient population at baseline, subjects who received AXA1125 experienced clinically and statistically significant improvement in mental (p=0.0097) and physical (p=0.0097) fatigue scores compared to placebo subjects and the results of this study are further described in a recently published paper by Finnigan et al.[90] The physical and mental fatigue scores are the two main components of the Chalder Fatigue Score utilized in the study. Further studies are needed to validate the improvements in patient-reported outcomes in a larger cohort of patients and the mechanistic drivers of improvement.

Conclusion

The long-term consequences of COVID-19 infection pose a significant burden to society

and the healthcare system, impacting the quality of life of affected patients and the associated support system. To address this pressing medical need, we at Axcella Therapeutics leveraged our systems biology platform to identify putative mechanisms of exertional fatigue due to long COVID, advancing the testing of our novel EMM composition of AXA1125 toward approval as a potential treatment option. Our preclinical and clinical evidence demonstrates that AXA1125 can potentially restore multiple essential mechanisms involved in mitochondrial biology, including improving bioenergetics and reducing inflammation and oxidative stress, and more work is needed to advance and validate the clinical improvements seen and the underlying mechanism of action. The composition could be a targeted treatment for exertional fatigue due to long COVID. In the future, novel research mechanisms are required to explore the factors contributing to variations in long COVID disease impact between individuals and across populations, mechanisms underlying the symptoms of long COVID, and the optimal treatment strategy for this debilitating condition. Moreover, subsequent studies are needed to validate the improvements in long COVID fatigue with AXA1125 and the underlying mechanism of action.

Conflict of Interest:

K.A. is an employee of Axcella Therapeutics and receives salaries and stock options from the company. M.K., M.R., J.P., R.W., and A.S. were employed by Axcella Therapeutics at the time of conduct of study-related activities. U.S. Patent and Trademark Office (USPTO) has granted U.S. Patent No. 11,737,999 with claims covering methods of use of Candidate AXA1125, for treating a subject having post-acute sequelae of COVID-19, also known as long COVID, particularly fatigue.

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Manuscript Contribution To The Field

Long coronavirus disease (COVID) 2019 or post-acute sequelae of COVID-19 is a syndrome wherein the symptoms of COVID-19 persist for over 12 weeks. Fatigue is one such long-lasting symptom observed in nearly 50% of patients with long COVID. Increasing evidence suggests the involvement of multiple biological pathways in the pathophysiology of long COVID, with mitochondrial dysfunction and inflammation playing critical roles in the associated fatigue. AXA1125 is a novel endogenous metabolic modulator comprising 5 amino acids (Leucine, Isoleucine, Valine, Arginine, and Glutamine) and an amino acid derivative (N-acetylcysteine). AXA1125 is a multi-target therapeutic that could potentially improve mitochondrial function by restoring cellular energetics and metabolism and reducing

oxidative stress and inflammation. Therefore, we hypothesize that AXA1125 may restore mitochondrial function and act as a potential treatment for fatigue associated with long COVID.

Author Contributions

K.A., J.P., M.K., A.S., R.W., AB: Reviewed and provided technical and editorial input.

K.A., J.P., and M.R.: Provided input into the development of the figures. All authors reviewed the manuscript.

Data Availability Statement

The original contributions outlined in the study could be provided by the corresponding author upon request. Any further inquiries can be directed to the corresponding authors.

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