Research Proposal and Findings

Spikeopathy- Spike Protein–Associated Pathobiology: A Series

I: Definition and Scope

"Spikeopathy" (1) is proposed here as a working, operational term to describe a spectrum of pathobiological states in which exposure to the SARS-CoV-2 spike glycoprotein—or spike-encoding genetic material—coincides with, plausibly contributes to, or appears to sustain a set of immune, vascular, autonomic, and neuroinflammatory abnormalities beyond the expected window of acute antigen clearance. The intent of this definition is not to assert a new disease entity prematurely, but to provide a scientifically testable framework that separates (i) spike-associated mechanisms from (ii) broader post-viral or post-inflammatory syndromes that may share clinical phenotypes yet arise through multiple upstream drivers.

At minimum, a Spikeopathy framework requires three components that can be interrogated empirically: exposure context, measurable host-response signatures, and a temporally and biologically coherent symptom/organ phenotype. Exposure context includes natural infection (acute COVID-19), post-acute sequelae of SARS-CoV-2 infection (PASC/Long COVID), and—where relevant—post-vaccination syndromes temporally associated with spike-encoding vaccines. Because spike is a shared antigen across these contexts, Spikeopathy is defined by mechanistic plausibility and biomarker-linked inference, not by social categories ("infection" versus "vaccine") or by symptoms alone.

Mechanistically, Spikeopathy is scoped to hypotheses in which spike (as protein, antigen fragments, immune complexes, or spike-bearing extracellular vesicles) could contribute to persistent or relapsing pathology through one or more of the following axes: endothelial activation and microvascular dysfunction, coagulation/platelet dysregulation and thromboinflammation, innate immune persistence with maladaptive cytokine and complement signaling, adaptive immune skewing including autoantibody generation, and neuroimmune effects mediated by barrier disruption, microglial priming, and autonomic network perturbation. Importantly, this scope does not presume that spike must be detectable in bulk plasma at all times; rather, it allows for low-abundance compartmentalization (tissue reservoirs, immune cell-associated antigen, endothelial interfaces, or vesicle-enriched fractions) that might escape conventional assays while still exerting biological effects.

Clinically, Spikeopathy is scoped to phenotype clusters commonly observed after SARS-CoV-2 exposure but not unique to it: exertional intolerance and post-exertional symptom exacerbation, dysautonomia (e.g., orthostatic intolerance/POTS-like presentations), cognitive dysfunction ("brain fog"), headaches and sensory disturbances, cardiopulmonary symptoms (palpitations, chest discomfort, dyspnea), and systemic inflammatory features (myalgia, sleep disruption, temperature dysregulation). These phenotypes are treated as downstream expressions of perturbations in microvascular perfusion, immune-neural coupling, and inflammatory signaling rather than as diagnostic proof. A Spikeopathy definition therefore explicitly requires careful differential diagnosis (e.g., thyroid disease, anemia, primary autoimmune disease, occult cardiopulmonary pathology, medication effects, deconditioning, and psychiatric comorbidity), because symptom overlap is extensive and misattribution would undermine both patient care and scientific credibility.

A key boundary condition is temporality. Spikeopathy pertains to abnormalities that persist or recur beyond expected acute convalescence and that demonstrate internal coherence across time—e.g., symptoms tracking with objective biomarker dynamics, reproducible physiologic signatures (tilt testing, cardiopulmonary exercise testing patterns, endothelial function measures), or consistent inflammatory/coagulation markers. However, temporality alone is insufficient; the framework prioritizes convergent validity: multiple independent measurement layers pointing toward the same biological process (for example, orthostatic symptoms plus autonomic testing abnormalities plus evidence of immune activation and endothelial perturbation).

In research terms, Spikeopathy is best treated as a testable model with graded certainty, not a binary label. A pragmatic stratification can be conceptualized: possible Spikeopathy (compatible phenotype with plausible exposure timing), probable Spikeopathy (phenotype plus supportive physiologic/immune-vascular signatures), and supported Spikeopathy (probable criteria plus direct evidence consistent with spike-related antigenic burden or spike-linked immune responses that map to pathophysiology). The highest tier demands rigorous assay validation: pre-analytic controls, orthogonal confirmation (e.g., immunoassay plus mass spectrometry or alternative antigen-capture methods), and careful interpretation to avoid contamination, cross-reactivity, or conflation of spike-specific signals with nonspecific inflammation.

Finally, the scope includes explicit negative space: Spikeopathy does not claim that spike is the dominant driver in all Long COVID or all post-vaccination illness, nor does it exclude other causal pathways such as viral persistence of non-spike components, latent virus reactivation, metabolic rewiring, microbiome-mediated inflammation, mast cell dysregulation, or structural organ injury from acute disease. Instead, Spikeopathy functions as a focused lens: it asks whether, in a defined subset, spike-associated biology is a meaningful upstream lever—one that can be measured, risk-stratified, and therapeutically targeted. The value of the framework will ultimately be judged by whether it improves explanatory power, enables reproducible subgrouping, and supports interventions that yield predictable, biomarker-aligned clinical benefit.

Here we begin with a series of research proposal topics starting with

1. Compartmentalization of Persistent SARS-CoV-2 Spike Protein in Long COVID: A Proposed Mechanism for Heterogeneous Symptomatology

References:

1. Khan, S. S., Catanzaro, J. A. (2023). "Peptide Mitigation as a Therapeutic Strategy for Spikeopathy: Addressing Aberrant Protein Signals Induced by mRNA Vaccines", Journal of Precision Biosciences, 5(1),1-10,10149

Title

Compartmentalization of Persistent SARS-CoV-2 Spike Protein in Long COVID: A Proposed Mechanism for Heterogeneous Symptomatology

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Abstract:

The diverse symptomatology of Long COVID (LC) presents a major clinical hurdle. We propose a novel hypothesis that the anatomical and functional compartmentalization of persistent SARS-CoV-2 Spike (S) protein in the blood underlies distinct LC symptom clusters. We postulate that the presence of S protein in different compartments—circulating free or bound state in serum, sequestered within formed element of the blood (blood cells) which include peripheral blood mononuclear cells (PBMCs), RBCs, platelets as well as extra cellular vesicles (EVs) —reflects unique pathological processes with direct clinical implications. The model discussed here focuses on detectable free serum S protein that may signify ongoing systemic viral persistence, potentially driving fluctuating, body-wide symptoms like fatigue, arthralgia, and fever, hypercoagulation, postural tachycardia immune dysregulation, brain-fog to mention a few of many symptoms of Long-COVID. The intracellular presence of S antigen within PBMCs could indicate a viral reservoir, leading to chronic antigenic stimulation and explaining relapsing-remitting symptoms and T-cell dysregulation. Most critically, we hypothesize that the packaging of S protein into EVs represents an active mechanism for targeted tissue injury; EV-associated S could facilitate cross-talk with the endothelium or neurons, thereby directly mediating specific symptoms such as autonomic dysfunction (e.g., POTS) and cognitive impairment. This compartmental framework provides a new lens through which we can interpret patient-specific biomarkers, suggesting that a patient's dominant symptom cluster may be predicted by the primary location of persistent S protein. Validating this model could stratify LC into mechanistically distinct phenotypes, paving the way for personalized diagnostics and targeted therapeutic interventions. This compartmental model paper will focus on S protein in the free serum, PBMCs and EVs. this to be followed by our forthcoming paper where we would be discussing it in context with RBCs, platelets, polymorphonuclear leukocytes (e.g., neutrophils/granulocytes) and immune complexes in the blood.

A. Introduction

The Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) pandemic, responsible for the COVID-19 illness, has left in its wake a complex and challenging public health crisis that extends far beyond the initial acute phase of infection (1). While the acute respiratory manifestations initially captured global attention, a significant proportion of convalescent individual’s report of debilitating symptoms, a condition now widely recognized as Post-Acute Sequelae of COVID-19 (PASC), or more commonly, Long COVID (LC) (2). This syndrome represents a multifaceted illness, with a spectrum of symptoms that can persist for weeks, months, or even years after the initial infection has resolved, creating a substantial burden on healthcare systems and deeply impacting patients' quality of life (3).

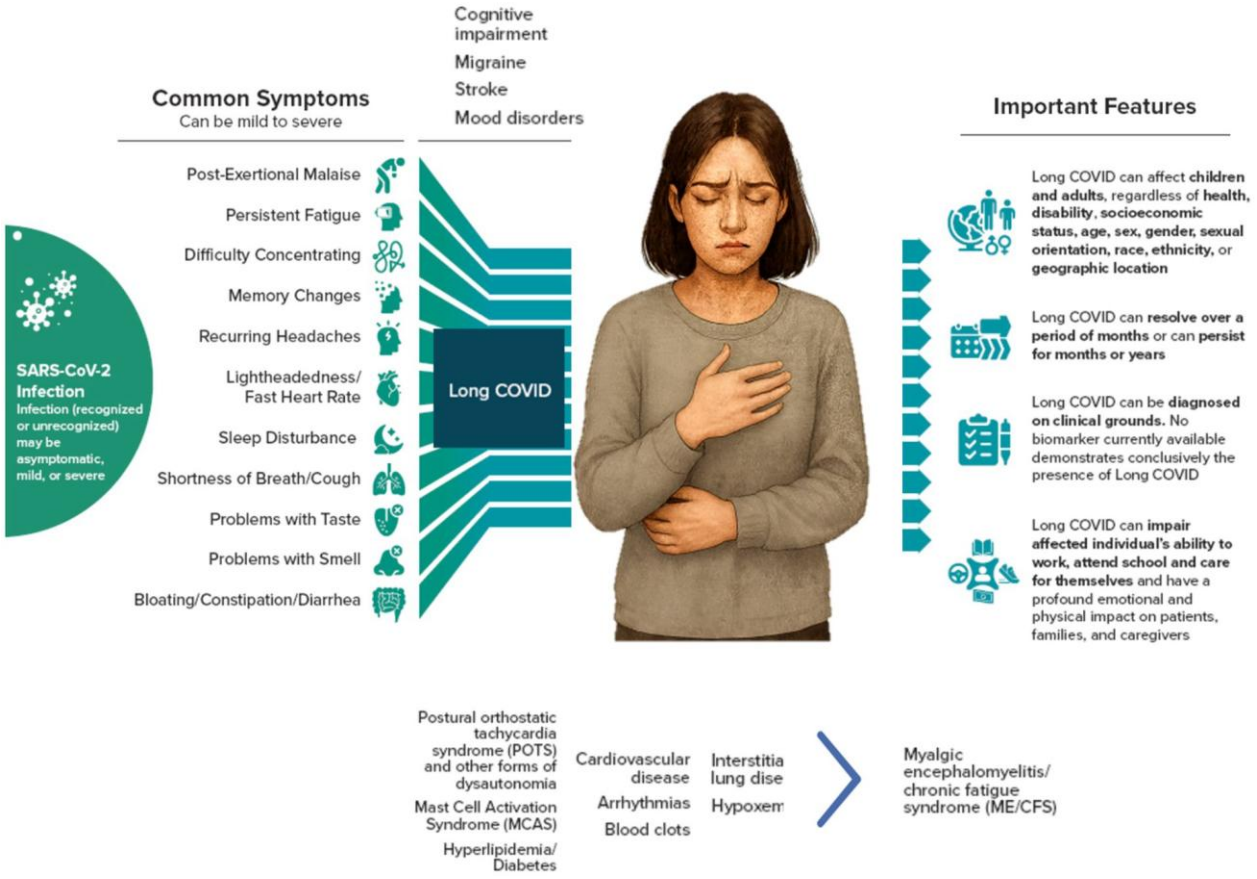


Figure1. A Clinical Overview of Long COVID Pathophysiology and Presentation.

This schematic summarizes the core concepts of Long COVID. **(A)** The condition can develop after any SARS-CoV-2 infection, regardless of initial severity. **(B)** It manifests through a constellation of common, often debilitating symptoms such as post-exertional malaise (PEM), cognitive impairment ("brain fog"), and dysautonomia. **(C)** Key clinical entities associated with Long COVID include POTS, ME/CFS—a distinct neuroimmune disease into which persistent Long COVID can evolve—and new-onset cardiovascular and metabolic conditions. **(D)** Critical features for clinicians include its widespread occurrence across all demographics, variable duration, profound functional impact, and the current reliance on clinical diagnosis in the absence of a specific biomarker.

The clinical presentation of Long COVID is remarkably heterogeneous, making it a diagnostic and therapeutic enigma for clinicians (Fig.1) (4). Patients frequently report a constellation of issues, including crushing fatigue that is worsened by exertion, cognitive impairment colloquially known as "brain fog," and a range of autonomic nervous system dysfunctions such as postural orthostatic tachycardia syndrome (POTS) (5). Other common complaints include persistent shortness of breath, chest pain, and musculoskeletal pain, creating a picture that does not fit neatly into any single pre-existing diagnostic category (6). This vast symptom diversity suggests that multiple, distinct pathological processes are likely at play, rather than a single unifying mechanism (7).

A leading theory to explain this persistence of symptoms is the presence of a viral reservoir or the continued presence of viral components within the host long after the primary infection (8). Among these components, the SARS-CoV-2 Spike (S) protein is of particular interest due to its pivotal role in viral entry into the host cells and its high immunogenicity (9). Several lines of evidence have now confirmed the detection of S protein antigens, and in some cases viral mRNA, in patients suffering from Long COVID, suggesting that the body's failure to fully clear the virus or its remnants may be a cornerstone of the pathophysiology (10). The mere presence of the S protein, however, does not adequately explain the wide range of clinical manifestations observed in the patient population.

This is where the concept of location of viral presence and production of S protein becomes critically important. The biological impact of a persistent antigen is not determined solely by its presence, but profoundly by its *context* and *compartmentalization* within the body (11). For instance, S protein circulating freely in the blood may elicit a very different immune response than one hidden inside a cell or one packaged for delivery to specific tissues in EVs. The human body is a landscape of distinct compartments, and the localization of the S protein within these compartments could hold the key to decoding Long COVID's symptomatology (12). We can begin to unravel this mystery by examining three key blood-based compartments: serum, peripheral blood mononuclear cells (PBMCs), and extracellular vesicles.

The serum, the cell-free liquid component of blood, represents a systemic compartment. The detection of S protein in serum would imply a state of active viral persistence at a remote site with the steady release of S protein and viral debris into the circulation (13). This freely

circulating antigen could continuously engage the immune system, leading to a state of chronic, body-wide inflammation. This persistent immune activation is a plausible mechanism for the generalized symptoms that afflict so many patients, such as profound fatigue, widespread pain, and recurrent fevers (14). In this model, the presence of serum S protein acts as a constant trigger, keeping the immune system in a heightened, and ultimately exhausting, state of alert.

In contrast, the presence of the S protein within peripheral blood mononuclear cells (PBMCs)—a heterogeneous group of immune cells including lymphocytes and monocytes—suggests a more covert and complex process (15). The intracellular sequestration of viral antigens points towards the establishment of a viral reservoir, where the virus or its components hide from humoral immunity. This reservoir could lead to chronic antigenic stimulation, potentially causing T-cell exhaustion or dysregulation, a phenomenon observed in other persistent viral infections (16). This relapsing and remitting pattern of viral activity could perfectly explain the fluctuating nature of symptoms many patients endure, where periods of relative wellness are interrupted by sudden "crashes" or flare-ups of illness (17). Though there is no or a little viral replication in the PBMCs by their presence in these cells clues towards an active role of these cells to tackle the S protein and attempts to clear it from the free circulating serum.

Perhaps the most intriguing and potentially targeted mechanism involves the packaging of the S protein into extracellular vesicles (EVs). EVs are small, membrane-bound particles released by cells that play a crucial role in intercellular communication (18). They can carry proteins, lipids, and nucleic acids from their parent cell and deliver this cargo to specific recipient cells. When a persistent S protein is loaded into these vesicles, it is no longer a passive floating antigen but becomes a guided missile (19). These EV-associated S proteins could be delivered directly to vulnerable tissues, such as myocardium, bone marrow and or neuronal cells, triggering localized inflammation and dysfunction without requiring systemic infection (20). This targeted delivery mechanism offers a compelling explanation for the specific organ-based symptoms of Long COVID, such as the cardiovascular dysregulation in POTS or the neuroinflammation underlying cognitive dysfunction (21).

Therefore, the central premise of this paper is that the anatomical and functional compartmentalization of the persistent SARS-CoV-2 S protein is a primary determinant of the heterogeneous clinical presentations in Long COVID. We propose a novel, compartment-based

model and examples wherein the presence of the S antigen in serum, PBMCs, or extracellular vesicles drives distinct pathological pathways, each correlating with a specific symptom cluster. By moving beyond the simple question of *if* the Spike protein is present, and instead focusing on *where* it is located, we can begin to stratify Long COVID into mechanistically distinct subgroups. This conceptual framework not only advances our pathophysiological understanding but also paves the way for the development of compartment-targeted diagnostics and personalized therapeutic strategies for this complex and debilitating condition.

B. Defining the Compartments: Proposed Sources and Pathogenic Roles of Persistent Spike Protein

To deconstruct the complex clinical puzzle of Long COVID, we propose a model centered on the anatomical and functional compartmentalization of the persistent SARS-CoV-2 Spike (S) protein. The specific location where the S protein resides—whether floating in the serum, hidden within immune cells, or packaged for delivery in extracellular vesicles—dictates its potential sources, its interaction with the host immune system, and ultimately, the clinical symptoms it may produce (12). This section delves into the proposed origins and pathogenic mechanisms for the S protein within each of these three critical compartments.

B1. Serum: The Systemic Inflammatory Driver

The serum represents the cell-free, liquid highway of the circulatory system. The detection of S protein in this compartment suggests a state of continual antigen exposure, where the protein circulates freely, unshielded from the body's immune surveillance (22). The proposed sources for this circulating antigen are multifaceted. One possibility is the ongoing, low-level replication of the virus in a protected tissue reservoir, such as the gut-associated lymphoid tissue or other immunoprivileged sites, which steadily sheds viral debris into the bloodstream (23). Another plausible source is the slow breakdown of infected cells that have harbored viral proteins for months, releasing their contents upon death (24).

Once in the serum (Fig2A), the S protein acts as a persistent immunological trigger. It can be recognized by circulating antibodies, potentially forming immune complexes (25). These

complexes can deposit in various tissues, including small blood vessels, and activate the complement system, a key arm of the innate immune response. This complement activation leads to localized inflammation, tissue injury, and the production of pro-inflammatory cytokines like IL-6 and TNF-α (26, 14). For the patient, this relentless, body-wide inflammatory state is not an abstract concept but a daily reality. It manifests as the profound, post-exertional fatigue that defines the lives of many, the diffuse muscle aches and joint pains that resist simple analgesics, and the low-grade fevers that signal an immune system stuck in a loop (27, 6). In this model, the serum S protein is the engine of a systemic fire, burning across the entire biological landscape.

B2. Peripheral Blood Mononuclear Cells (PBMCs): The Intracellular Reservoir

In stark contrast to the public circulation of the serum, the intracellular environment of PBMCs offers a hidden sanctuary for the S protein. The presence of S antigen within these cells—particularly monocytes and macrophages—points toward a more covert and complex pathological process (Fig2B) (15, 28). The source here is likely the initial infection of these very cells. Monocytes and macrophages, key players in the innate immune system, express the ACE2 receptor and other co-factors that can facilitate SARS-CoV-2 entry, allowing the virus to establish a foothold within the body's own defense network (29). Even if full viral replication does not occur, these cells can engulf virus-infected cells or debris, leading to the persistent presence of the S protein within their cytoplasm (Fig2B) (30).

The pathogenic consequences of this intracellular sequestration are significant. The immune system is adept at surveying the blood for foreign invaders, but it struggles to detect pathogens that have learned to hide inside cells. The presence of S protein within antigen-presenting cells, such as monocytes, can lead to chronic, dysregulated antigen presentation (31). This can, in turn, exhaust T-cells, the orchestrators of adaptive immunity, rendering them less effective and potentially driving the lymphopenia observed in some Long COVID patients (16). This state of immune dysregulation creates a biological rollercoaster for the patient. The relapsing-remitting nature of their symptoms—the "crashes" that follow periods of overexertion or stress—can be understood as episodic flares of antigen presentation and T-cell activation (17, 32). Furthermore, the inflammatory signals released by these chronically activated immune cells can cross the blood-brain barrier, potentially activating microglia and disrupting neural circuits, providing a

plausible mechanism for the debilitating "brain fog" and cognitive deficits that are a hallmark of the condition (33).

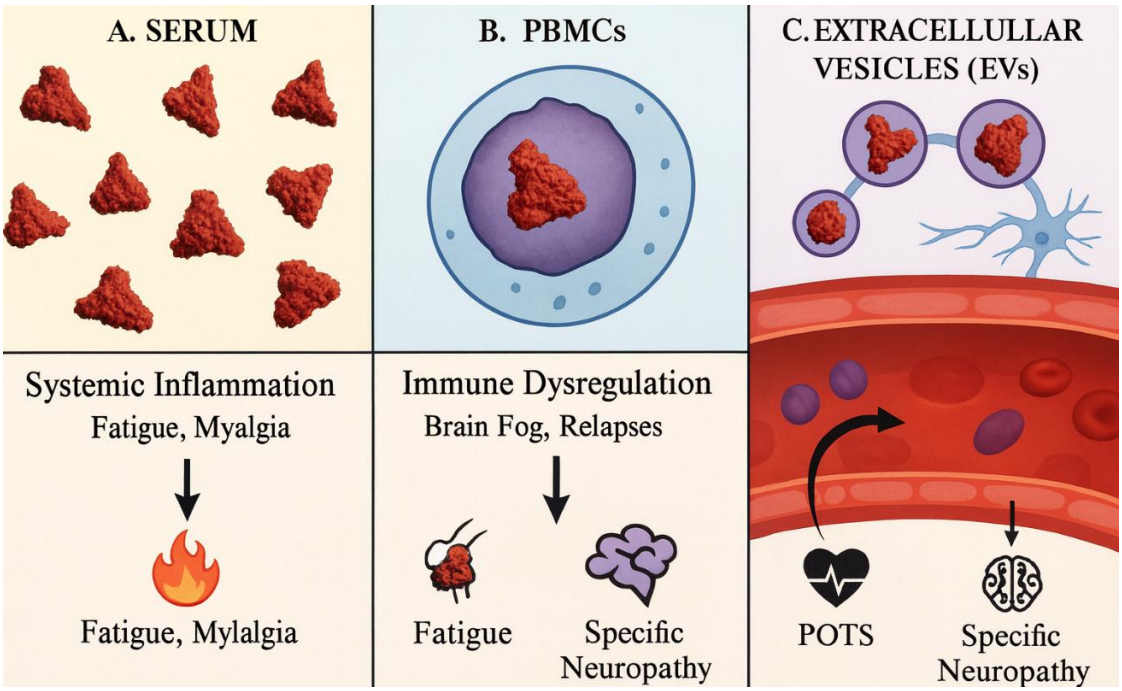


Figure2. The Compartment-Based Concept for Long COVID Pathogenesis.
This schematic illustrates the proposed mechanism by which the persistence of the SARS-CoV-2 Spike protein in distinct biological compartments drives specific pathological processes and symptom clusters. **(A) Serum Compartment:** Circulating Spike protein acts as a continual trigger for systemic inflammation, manifesting as widespread symptoms like fatigue and myalgia. **(B) PBMC Compartment:** Intracellular sequestration of Spike protein within peripheral blood mononuclear cells (e.g., monocytes) leads to chronic immune dysregulation and T-cell exhaustion, underlying neuroimmune symptoms such as cognitive dysfunction ("brain fog") and a relapsing-remitting course. **(C) Extracellular Vesicle (EV) Compartment:** Spike protein packaged into EVs facilitates targeted tissue injury; upon delivery to specific cell types (e.g., endothelial or neural cells), it can disrupt normal function, leading to focal conditions like postural orthostatic tachycardia syndrome (POTS) and other forms of autonomic neuropathy.

B3. Extracellular Vesicles (EVs): The Targeted Messengers

Perhaps the most sophisticated and targeted mechanism involves the packaging of the S protein into extracellular vesicles. EVs are natural lipid-bound nanoparticles released by almost all cell types, acting as a fundamental system for intercellular communication by shuttling proteins, lipids, and nucleic acids between cells (18, 34). The proposed source of EV-associated S protein is the very cells that are persistently infected or have ingested viral material. During the biogenesis of these vesicles, the S protein can be incorporated into the vesicle membrane or enclosed within its lumen (Fig2C) (35). This process transforms the S protein from a passive antigen into an active, protected cargo, ready for delivery.

The pathogenic role of these EV-loaded S proteins is particularly insidious. They function as Trojan horses, enabling the antigen to bypass neutralizing antibodies in the serum and deliver its cargo directly to the surface of recipient cells (19, 36). For example, an EV carrying the S protein on its surface can bind to the ACE2 receptor on an endothelial cell lining a blood vessel. This binding can trigger a localized inflammatory response within the endothelium, promoting blood clot formation and impairing vascular tone (20, 37). This targeted endothelial injury offers a direct mechanistic pathway for the cardiovascular symptoms prevalent in Long COVID, such as palpitations and the dramatic orthostatic intolerance seen in POTS (5). Similarly, if these vesicles cross the blood-brain barrier or are released by glial cells within the central nervous system, they could deliver the S protein to neurons, triggering neuroinflammation and neuronal dysfunction that underlies not only cognitive impairment but also conditions like anosmia and parosmia (38, 21). The patient's experience of specific, organ-focused symptoms—a racing heart upon standing, a malfunctioning sense of smell, or a sharp decline in executive function—may be the direct result of these precisely delivered molecular messages.

In summary, this compartment-based model provides a structured framework to move from the vague notion of "Spike persistence" to a set of testable, mechanistic hypotheses. Each compartment—serum, PBMC, and EV—represents a unique pathogenic niche with distinct sources and consequences, offering a coherent explanation for the bewildering heterogeneity of Long COVID. By identifying which compartment is dominant in a given patient, we may finally be able to stratify this complex syndrome into manageable biological subgroups.

C. A Proposed Methodological Framework for Isolating and Validating Compartment-Specific Biomarkers

Translating the compartment-based hypothesis into a tangible research program requires a rigorous and reproducible methodological framework. To move from theory to actionable data, we propose a multi-stage approach designed to isolate, quantify, and contextualize the persistent Spike (S) protein within the serum, peripheral blood mononuclear cells (PBMCs), and extracellular vesicle (EV) compartments from well-phenotyped Long COVID patients. The ultimate goal of this framework is not merely to detect the S protein, but to establish a clear, causal link between its specific location and the patient's clinical reality, thereby validating the core tenets of our model (12, 22). This section outlines a comprehensive strategy to achieve this, focusing on technical precision and clinical correlation.

The foundation of this entire endeavor is the establishment of a deeply characterized patient cohort. We propose recruiting a minimum of 150 individuals with a confirmed Long COVID diagnosis according to the World Health Organization criteria, alongside a control group of 50 individuals who recovered fully from acute COVID-19 without persistent symptoms (2, 39). The power of this study will lie in the granularity of clinical data. Each participant will undergo a standardized assessment battery that goes far beyond a simple symptom checklist. This includes detailed neuropsychological testing for cognitive function, active stand tests or tilt-table testing for objective assessment of POTS, and validated patient-reported outcome measures for fatigue, pain, and quality of life (5, 27). This rich phenotypic tapestry is essential, as it will allow us to move from vague associations to precise correlations between biomarker location and specific symptom clusters.

Beginning with the serum compartment, the isolation and analysis are relatively straightforward but require high sensitivity. Blood will be collected in serum-separating tubes, allowed to clot, and centrifuged to obtain clear serum aliquots (Fig3) (40). The detection of the S protein, and particularly its S1 subunit which is shed upon viral entry, will be performed using a combination of techniques. By employing ultrasensitive Single Molecule Array (Simoa) technology, which can detect proteins at sub-femtogram per milliliter concentrations, far below the limit of conventional ELISA kits (41). To distinguish between free-floating antigen and potential immune complexes, serum samples will also be treated with a dissociating agent prior to analysis, a step

that can significantly increase detectable levels and provide insight into the antigen's bioavailability (25). This multi-faceted approach to serum analysis will help clarify whether it is a primary driver of pathology or a secondary consequence of another process.

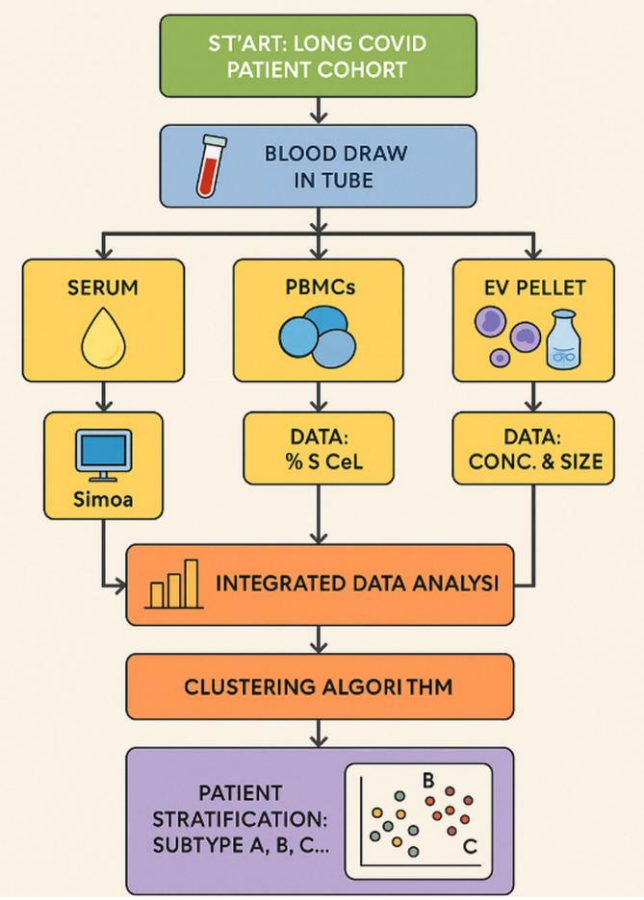


Figure 4. A Translational Pipeline for Stratifying and Treating Long COVID. This conceptual workflow outlines the proposed pathway from diagnosis to personalized treatment based on the compartmentalization of persistent SARS-CoV-2 Spike (S) protein. The process begins with a clinical diagnosis of Long COVID (LC). The critical diagnostic stratification involves profiling the S protein across three blood compartments: serum, peripheral blood mononuclear cells (PBMCs), and extracellular vesicles (EVs). The dominant compartment assigns a patient to a specific biological subtype (I, II, or III). This stratification directly informs targeted therapeutic strategies: Subtype I (systemic inflammation) would receive immunomodulators; Subtype II (intracellular reservoir) would undergo therapies to enhance viral clearance; and Subtype III (EV-mediated injury) would be treated with EV biogenesis or uptake blockers. The ultimate goal of this precision medicine approach is to achieve significantly improved clinical outcomes.

The investigation of the PBMC compartment requires a more nuanced approach to isolate the specific cellular carriers. Fresh blood will be collected in anticoagulant tubes, and PBMCs will be isolated using a standard Ficoll-Paque density gradient centrifugation protocol (Fig3) (42). The critical next step, however, is to move beyond a homogenized PBMC population. The use of fluorescence-activated cell sorting (FACS) can meticulously separate key subpopulations, particularly classical (CD14++ CD16-), intermediate (CD14++ CD16+), and non-classical (CD14+ CD16++) monocytes, as these cells have been implicated as potential viral reservoirs (28, 29). The presence of the S protein within these sorted cells will be probed using intracellular flowcytometry and, with greater sensitivity, by lysing the cells and performing the same Simoa assays used for serum. Detecting the protein inside a specific myeloid cell type would provide powerful evidence for its role as a persistent intracellular reservoir driving chronic immune dysregulation.

The most technically challenging, yet potentially most revealing, compartment is that of the extracellular vesicles. A major pitfall in EV research is the co-isolation of contaminants like lipoproteins or protein aggregates. Therefore, we propose a rigorous, sequential isolation protocol to ensure purity (34). First, larger particles and dead cells will be removed from platelet-poor plasma through a series of low-speed centrifugations and 0.22-micron filtration. The EVs will then be isolated using size-exclusion chromatography (SEC), which separates particles based on their hydrodynamic radius and is renowned for preserving vesicle integrity and function (43). To confirm the successful isolation of EVs, a nanoparticle tracking analysis can be used to determine the size and concentration profile, and Western blotting for canonical EV markers such as CD9, CD63, and CD81, while excluding negative markers like Apolipoprotein B (44).

Once a pure EV population is secured, the crucial question becomes: Is the S protein merely co-isolated with the vesicles, or is it genuinely packaged within or on them? To answer this, the use of several techniques are proposed. Detergent treatment of the EV preparation will help distinguish between surface-presented and luminal S protein (Fig3) (45). Most importantly, we suggest to utilize immuno-electron microscopy, a technique that uses gold-labeled antibodies to visually confirm the precise location of the S protein on or within the vesicle membrane, providing incontrovertible proof of its association with these nanoparticles (46). This level of validation is critical for supporting the "Trojan horse" hypothesis of EV-mediated pathogenesis.

The final and most critical phase of the framework is the integrative analysis. Simply having data from three separate compartments is insufficient; the power lies in their convergence. Application of advanced multivariate statistical models, such as unsupervised clustering algorithms, to determine if the quantitative and qualitative data from the three compartments naturally group patients into distinct biological subgroups, as has been reported before (47). The central hypothesis can be tested by constructing correlation matrices to see if, for instance, high levels of EV-associated S protein are a stronger predictor of POTS than serum S protein, or if PBMC-S protein load correlates more tightly with cognitive test scores than the other compartments. This systematic, multi-compartment profiling, anchored to deep clinical phenotyping, will provide the evidence needed to transition our model from a compelling narrative to a validated, clinically useful tool for stratifying Long COVID and guiding future targeted therapies.

D. Broader Implications and Future Directions

The compartment-based model for Long COVID, if validated, extends far beyond a simple academic exercise. It offers a tangible roadmap to fundamentally reshape our clinical approach to this complex syndrome, moving it from a diagnosis of exclusion to one grounded in measurable biology. By providing a structured framework to deconvolute the profound heterogeneity of the condition, this model opens the door to a new era of personalized medicine for millions of affected individuals worldwide (3, 7). The implications are profound, touching upon diagnostics, therapeutics, and even our understanding of other post-viral illnesses, promising to replace the current fog of uncertainty with a clear path forward.

The most immediate and transformative application lies in the realm of diagnostics. Currently, a Long COVID diagnosis is primarily based on patient history and the exclusion of other conditions, a process that is often lengthy, frustrating, and inherently subjective for both the patient and the clinician (2, 4). The validation of compartment-specific biomarkers would allow for the development of a definitive blood test. Imagine a clinical assay that not only confirms Long COVID but can stratify a patient into a specific subgroup—for instance, a "Systemic Inflammatory" profile (high serum S), a "Covert Immune" profile (high PBMC S), or a "Targeted Neurovascular" profile (high EV S) (48). Such a test would provide much-needed validation for patients who often face skepticism and would empower clinicians to make confident diagnoses, effectively ending the diagnostic odyssey that characterizes so many patients' journeys today.

This biological stratification naturally paves the way for rationally designed, targeted therapeutic interventions, moving away from the current one-size-fits-all approach. For patients in the "Systemic Inflammatory" subgroup, the logical therapeutic strategy would involve targeted immunomodulation. This could include monoclonal antibodies against key cytokines like IL-6 or IL-1β, or even the cautious use of antiviral regimens designed to clear the presumed residual viral reservoir fueling the antigenemia (26, 49). For those with the "Covert Immune" profile, where the S protein is hidden within PBMCs, therapies might focus on agents that can enhance intracellular viral clearance or reset the exhausted immune system. Drugs that target autophagy or specific aspects of antigen presentation could be explored to silence the persistent internal trigger (31, 50). This shift from symptomatic management to mechanism-based treatment represents the holy grail of modern medicine (Fig4).

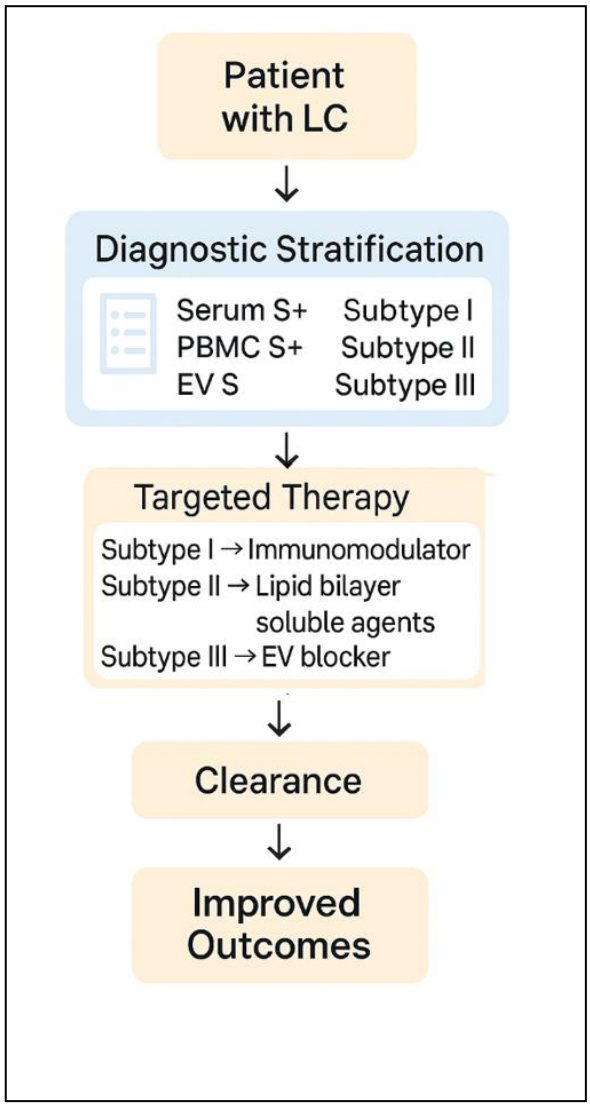


Figure 4

Proposed framework for a biomarker-driven, personalized medicine approach to Long COVID (LC). This model transitions LC from a diagnosis of exclusion to one based on measurable biological signals. Patients with LC undergo **diagnostic stratification** through biomarker screening across serum, PBMCs (peripheral blood mononuclear cells), and extracellular vesicles (EVs), resulting in classification into three biologically defined subtypes. Each subtype is then matched to a **targeted therapy** — Subtype I with immunomodulators, Subtype II with strategies that can act across lipid bi-layer, and Subtype III with EV blockers and agents targeting the expressed S-protein — aiming to improve patient outcomes. This structured roadmap enables a more precise and personalized clinical approach to address the heterogeneity of LC

The most complex, yet potentially most rewarding, challenge involves treating the "Targeted Neurovascular" subgroup. Here, the proposed pathogen is not just a protein, but a delivery system. Therapeutic strategies would need to focus on intercepting the harmful cargo. This could involve developing apheresis techniques specifically designed to remove EV populations from the blood or drugs that inhibit the formation or release of EVs from parent cells (37, 51). Furthermore, understanding the "zip code" of these vesicles—what makes them target specific tissues—could lead to therapies that block their uptake in vulnerable organs like the endothelium or the brain, effectively neutralizing the Trojan horse before it can deliver its damaging payload (20, 52).

The implications of this model also ripple outward, offering a new lens through which to view other enigmatic chronic conditions. The similarity between Long COVID and myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) is striking and has been widely noted (27, 32). It is plausible that a similar compartmentalization of a persistent viral antigen—perhaps from Epstein-Barr virus or other common pathogens—could underpin the symptomatology of ME/CFS and other post-infectious illnesses (53). Our proposed methodological framework could be directly applied to these conditions, searching for compartment-specific biomarkers that have thus far remained elusive. In this way, the tragic COVID-19 pandemic may inadvertently provide the key to unlocking mysteries that have confounded medicine for decades.

To bring this vision to fruition, several critical future directions must be pursued. First, large-scale, longitudinal cohort studies are essential to validate the initial correlations between compartments and symptoms and to understand how these biomarker profiles evolve over time (54). Does the dominant compartment shift, or do patients remain in a stable subgroup? Second, robust *in vitro* and animal models must be developed to move from correlation to causation. For example, can injecting EVs isolated from Long COVID patients into model organisms recapitulate aspects of POTS or cognitive dysfunction (55)? Finally, this research must be integrated with advanced omics technologies. Correlating the compartment data with proteomic, metabolomic, and autoantibody profiles from the same patients would paint an even more comprehensive picture of the underlying pathophysiology, revealing the full cascade of events from the persistent S protein to the patient's lived experience (56).

The compartmentalization hypothesis provides more than just an explanation; it provides a catalyst for change. It argues that the heterogeneity of Long COVID is not a barrier to understanding, but rather a clue that points toward distinct biological endotypes waiting to be discovered. By embracing this complexity and systematically investigating the specific niches where the SARS-CoV-2 Spike protein persists, we can transition from a state of therapeutic paralysis to one of targeted action. The path forward is clear: to refine these biomarkers, validate them in diverse populations, and usher in a future where a Long COVID diagnosis comes with not just a label, but a precise biological profile and a personalized plan for restoration and recovery.

E. Targeting the S Protein in compartments

There have been a very few attempts to target the S protein in the aforesaid compartments and relate them with the clinical outcome in form of particular symptoms being improved in patients. Some experimental findings have been shown to target the S protein in the above-mentioned compartments with combination of natural compounds Fig.5a-b which resulted in reduction of the Spike load in PBMCs and EVs, (57) with symptomatic improvements in the patients. Clinical trails with these medicinal products and other know S protein targeting or digesting medication are needed, corelating them with symptomatic improvements can help validate and evaluate the concept of targeting S protein containing compartments. Also, the reduction in the S protein levels after using herbal products or other medications used to target the S protein could be due

an anti-viral effect of the compounds used. Reduction in the N-antigen along with the reduction in S protein could serve a biomarker for viral replication with S protein production being targeted improving the clinical outcome in LC patients.

Patient -X

22.07.2024

Spikeprotein in Plasma/Serum	**NEGATIV**
Spikeprotein in Exosomen	**NEGATIV**
Spikeprotein in Immunzellen (PBMC)	**POSITIV 188,04 pg/2,5x10^6 Zellen**
LINE-1 in Immunzellen (PBMC)	**NEGATIV**
SARS-Cov-2 RNA in Immunzellen (PBMC) (Persistenz)	**NEGATIV**

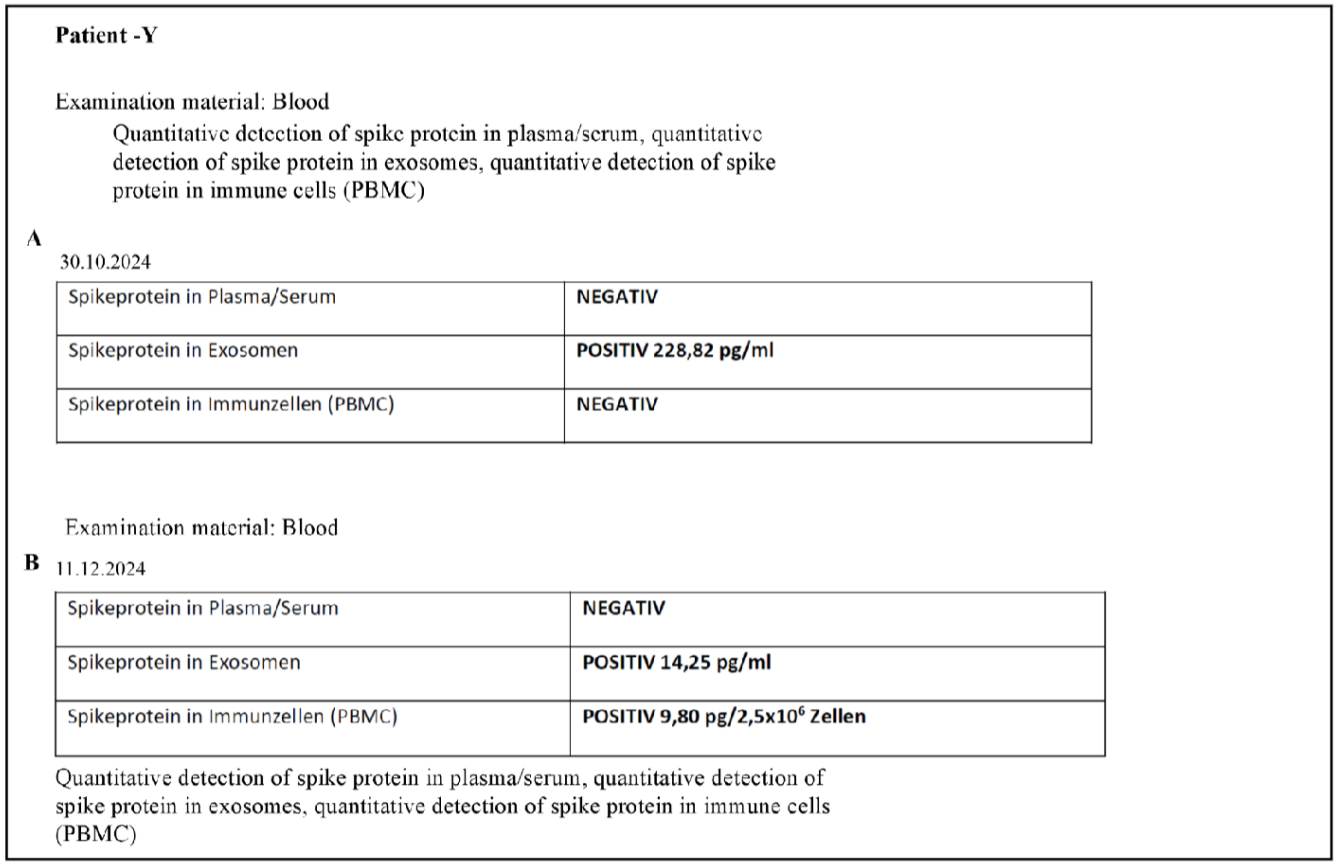
A 24.09.2024

Spikeprotein in Plasma/Serum	**NEGATIV**
Spikeprotein in Exosomen	**NEGATIV**
Spikeprotein in Immunzellen (PBMC)	**POSITIV 17,28 pg/2,5x10^6 Zellen**

B 30.10.2024

Spikeprotein in Plasma/Serum	**NEGATIV**
Spikeprotein in Exosomen	**NEGATIV**
Spikeprotein in Immunzellen (PBMC)	**POSITIV 5,18 pg/2,5x10^6 Zellen**

***Figure 5a.** Shows values of Spike protein in PBMC in July 2024 in Patient X (Top panel) compared to the reduction observed in subsequent months (A-September, B-October 2024) after treatment with a formulation composed of nutraceutical known as Vedicinals®9.Notably, the patient's neurological symptoms, particularly his ability to concentrate, showed marked improvement. By November 2024, sustained benefits were observed with continued Vedicinals9® therapy. [* with permission of Joachim Gerlach et al, ref.57]



Patient -Y

Examination material: Blood

Quantitative detection of spike protein in plasma/serum, quantitative detection of spike protein in exosomes, quantitative detection of spike protein in immune cells (PBMC)

A

30.10.2024

Spikeprotein in Plasma/Serum	**NEGATIV**
Spikeprotein in Exosomen	**POSITIV 228,82 pg/ml**
Spikeprotein in Immunzellen (PBMC)	**NEGATIV**

Examination material: Blood

B 11.12.2024

Spikeprotein in Plasma/Serum	**NEGATIV**
Spikeprotein in Exosomen	**POSITIV 14,25 pg/ml**
Spikeprotein in Immunzellen (PBMC)	**POSITIV 9,80 pg/2,5x10^6 Zellen**

Quantitative detection of spike protein in plasma/serum, quantitative detection of spike protein in exosomes, quantitative detection of spike protein in immune cells (PBMC)

***Figure 5b**. Shows values of Spike protein in PBMC in July 2024 in patient Y (A) compared to the reduction observed in subsequent months (B) after treatment with a formulation composed of nutraceutical known as Vedicinals9® the patient's symptoms, particularly his POTS and symptoms related to hypercoagubility, showed marked improvement. [* with permission of Joachim Gerlach et al, ref.57]

F. Discussion

The model we have presented—that the clinical chaos of Long COVID can be navigated using a compass of compartmentalization—offers a structured and testable framework to address one of the most pressing medical challenges of our time. Throughout this paper, we have built a case that the persistent SARS-CoV-2 Spike protein is not a monolithic entity but a pathogen whose influence is dictated by its precise location within the host (12, 22). By tracing its presence to the systemic reservoir of serum, the hidden sanctuary of PBMCs, or the targeted delivery system of extracellular vesicles, we can begin to map the bewildering array of symptoms onto distinct, mechanistic pathways. This synthesis moves the conversation beyond the simple question of persistence and toward a more nuanced understanding of pathological context, providing a much-needed biological narrative for a condition often described in purely experiential terms (4, 48).

It is crucial, however, to situate our compartment-based hypothesis within the wider landscape of proposed Long COVID mechanisms, not as a replacement, but as a potential integrator. For instance, the widespread observation of autoantibodies in Long COVID patients is a compelling parallel pathology (25, 58). Our model could provide a trigger for this autoimmunity; the persistent presentation of the S protein, especially within the lymphoid environment or via EVs mimicking self-antigens, could drive a breakdown in immunological tolerance over time (19, 58). Similarly, the documented issue of microthrombi and endothelial dysfunction in these patients fits seamlessly into our framework (37, 60). The EV-associated S protein, in particular, represents a direct vector for delivering a pro-inflammatory and pro-thrombotic signal to the vascular endothelium, offering a plausible mechanism for the localized vascular injury that other researchers have observed (20, 52). Therefore, our model does not dismiss these findings but rather offers a potential upstream source, connecting a persistent viral trigger to its diverse downstream consequences.

Naturally, this hypothetical framework is not without its limitations, and acknowledging them is essential for its rigorous future testing. The technical challenges are significant, particularly in the

isolation and characterization of extracellular vesicles. The field of EV research is fraught with the perils of co-isolating non-vesicular contaminants, and standardizing these protocols across laboratories will be a hurdle that must be overcome to ensure reproducible results (43, 61). Furthermore, the patient population for Long COVID is notoriously heterogeneous, and our proposed subgroups may themselves contain further layers of complexity. A patient's pre-existing immune status, genetic background, and the specific viral variant of their initial infection are all confounding variables that could influence which compartment becomes the dominant reservoir (62, 63). Our model simplifies this complexity to provide a starting point, but it must be stress-tested against these real-world variables.

The journey from this theoretical model to clinical application is long and requires a steadfast commitment to translational research. The proposed methodological framework provides a starting point, but validating it will require global, multi-center collaborations to recruit the large, diverse cohorts necessary to ensure the findings are generalizable (54, 64). Beyond mere correlation, the next critical step is to establish causation. Can we, for example, take EVs isolated from a patient with severe POTS and recapitulate aspects of cardiovascular dysregulation in an animal model? (55, 65). Such experiments are ethically and technically complex but are the bedrock upon which a true mechanistic understanding is built. They would transform a compelling correlation into a proven causal relationship, solidifying the role of EV-mediated pathogenesis.

Ultimately, the most promising aspect of this compartment-based view is its inherent flexibility. As new research emerges, the model can be refined and expanded. Perhaps future studies will reveal that the specific mutations in the S protein of different variants predispose it to favour one compartment over another, explaining the differences in Long COVID risk between infections with, for instance, Delta versus Omicron variants (66, 67). The framework also invites integration with other omics technologies. Imagine correlating a patient's dominant compartment with their unique autoantibody profile, their gut microbiome composition, or their T-cell receptor repertoire (56, 68). This multi-dimensional approach would move us from stratified subgroups toward truly personalized medicine, where a patient's treatment is guided by a complete biological signature, not just a single biomarker.

G. Conclusion

In the face of a condition as complex and debilitating as Long COVID, the medical and scientific community has often struggled to find a clear path forward. The sheer heterogeneity of the patient experience has been a significant barrier, making it difficult to design targeted trials and leaving patients with a sense of being medically abandoned. The compartmentalization hypothesis we have detailed throughout this paper offers a way to cut through this complexity. It proposes that the very heterogeneity that defines Long COVID is not noise to be ignored, but a signal to be decoded—a direct reflection of the specific biological niche where the SARS-CoV-2 Spike protein has taken up long-term residence.

This is more than just a theoretical exercise; it is a call to action and a paradigm shift. It argues that we must stop viewing Long COVID as a single illness and begin the meticulous work of identifying its mechanistically distinct subtypes. By focusing our investigative lenses on the serum, the PBMC, and the extracellular vesicle, we can transform an overwhelming clinical problem into a set of solvable scientific questions. Additionally, the various methods adopted to make the circulation free of the spike from these compartments by various types of Apheresis including H.E.L.P apheresis can be tested for its efficacy (69). The roadmap is now clear: to rigorously validate these compartment-specific biomarkers, to unravel the precise molecular pathways they initiate, and to translate this knowledge into diagnostic tests that provide validation and therapies that deliver hope. For the millions navigating the uncertain terrain of Long COVID, this model lights a path out of the fog, toward a future where their diagnosis comes with not just a label, but a clear and personalized plan for reclaiming their health

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