

CG-MS750: A New Therapeutic Paradigm for Multiple Sclerosis

Project Concept for the Development of a Genetic Pharmaceutical for Multiple Sclerosis (MS) Therapy.

The overall concept of the project is based on a comprehensive integration of traditional elements, which have stood the test of time, and innovative components that leverage the latest advancements in the field, all meticulously combined to create a supra-additive effect that delivers a unique, cutting-edge solution. Within this concept, the following components are included:

1. **Focus on Pathology:** Priority attention is given to the disposal of pathological intracellular protein aggregates and to the processes of neuroregeneration.
2. **Multiplicity of Targets:** Therapeutic versatility is achieved through the use of a composition of genes responsible for a range of biological processes — including the disposal of pathological protein aggregates, neuron restoration, and the regeneration of synaptic connections.
3. **Unique DNA Vectors:** Our therapeutic DNA vectors of VTvaf17 and GDTT1.8NAS series developed and patented as a platform solution, elegantly combine efficacy, safety, and the flexibility to vary with universal and tissue-specific promoters and coding sequences of target genes.
4. **Precision Expression:** The use of combinations of tissue-specific and inflammation-activated promoters within DNA vectors guarantees precise and efficient expression of target genes in specific cells, enhancing overall therapeutic efficacy and minimizing ectopic effects. The application of functionalized microspheres "DNA + Liposome + PEG" ensures affinity to target cells and contributes to achieving maximum efficacy while reducing aberrant effects.
5. **Advanced Delivery Systems:** A complex of cationic liposomes plus PEG functionalized with specialized reagent has been selected as the delivery system, which ensures the achievement of therapeutically significant concentrations of target proteins, thereby maximizing the drug's therapeutic effect.
6. **Use of Native Genes:** The incorporation of only natural human genes into the medicinal product ensures harmonious integration of the drug's action with natural biological processes, reducing the risk of adverse reactions and enhancing the drug's biocompatibility.
7. **Regulatory Compliance:** The composition of structural elements of the vectors, developed and patented as part of our platform solution, fully complies with FDA and EMA requirements, guaranteeing adherence to strict safety and efficacy standards.
8. **Technological Efficiency.** The use of proven technologies allows the drug to be manufactured at various standard biotechnological facilities, achieving both competitive pricing and high profitability.

Such an integrated combination of traditional and innovative approaches enables the development of a unique drug for MS therapy with a supra-additive effect that exceeds the sum of individual components. This solution ensures high efficacy and safety of treatment, meeting modern medical needs.

Components and Stages of Developing a Pharmaceutical for Multiple Sclerosis Therapy:

I. Strategy Development

II. Therapeutic Implementation

III. Description of Tools

IV. Gene Selection

V. Delivery Methods

VI. Treatment Protocols

VII. Economic Feasibility

I. Strategy Development

To develop a reliable strategy it is proposed to use only proven approaches, facts, arguments in the project, i.e. to confirm experimentally the relationship between dynamic changes in biological indicators with changes in the expression level of target genes.

Multiple sclerosis is a multifactorial condition, and the development of a successful therapy requires not only considering the full spectrum of biological disturbances (factors) associated with the disease but also ranking them by significance with a mandatory assessment of the scientific and experimental evidence pertaining to each.

The pathogenetic concept adopted in this project accords with mainstream understanding: the cognitive impairments, sensory deficits, coordination and balance problems, visual disturbances, and other manifestations of MS result from a malfunctioning immune system that attacks the body's own tissues—namely, the myelin sheath of neurons. This demyelination slows or completely disrupts signal conduction along neural pathways, giving rise to the wide spectrum of neurological symptoms. As the disease progresses, neuronal loss and the breakdown of neural networks lead to severe disability.

Aspects related to halting the autoimmune process and reducing inflammation are not treated as priorities in the present formulation; it is assumed that these elements of MS will be managed with currently available high-efficacy drugs—baseline immunotherapies (immunosuppressants), glucocorticoids, monoclonal antibodies that block immune-cell entry into the CNS, sphingosine-1-phosphate receptor modulators, and similar agents that mitigate inflammation via immune modulation.

Likewise, remyelination and regeneration of damaged structures in the peripheral nervous system (PNS) are regarded as lower-priority goals, since PNS neurons are affected far less frequently in MS. Even so, given the overlap in underlying biology, the proposed therapy is expected to confer some ancillary benefit to PNS neurons.

The strategy of this project is focused on the development of a medicinal product targeting biological processes directly implicated in the pathogenesis of MS for which no currently approved therapeutic agents exist:

1. modulation of glial scars that have formed in CNS regions subjected to pathological insult;

2. regeneration of the damaged target CNS areas, remyelination of axons of neurons within those affected CNS regions

A detailed rationale underpinning the biological processes and the therapeutic priorities is provided in the following table:

Disease Manifestation (Biological Process), Pathogenesis	Therapeutic Task	Inclusion of Therapeutic Task in Project Strategy	Comment
Immune-cell attack on the neuronal myelin sheath; inflammation	Suppression of autoimmune processes and minimisation of inflammation	No	This aspect is not prioritised, as it can be managed with existing marketed therapies. Any anti-inflammatory regimen will need to be harmonised with the dosing schedule of the investigational product.
Formation of glial scars in affected CNS regions	Modulation of glial scars in the damaged CNS areas while simultaneously protecting partially injured yet recoverable neurons from phagocytosis	Yes	Glial-scar modulation is a key therapeutic challenge in multiple sclerosis and currently lacks universally accepted treatments.
Formation of glial scars in affected PNS regions	Modulation of glial scars in the damaged PNS areas	No	This aspect is not prioritised because PNS neurons are affected far less frequently in MS. Limited positive effects may still arise indirectly from the project's core therapeutic activities.
Damage and death of neurons	Regeneration of injured neurons; neurogenesis of new neurons	Yes	Neurons damaged by immune attack clearly require regeneration to restore function. Neurogenesis is also important, though its activation is constrained by the reduced pool of neuronal stem cells.
Demyelination of neurons due to immune attack	Remyelination of damaged neurons	Yes	Remyelination is one of the critical therapeutic challenges in MS and presently has no universally accepted solutions.

II. Therapeutic Implementation

The therapeutic implementation (Integral Task) applied in this project consists of formulating therapeutic stages, incorporating into each of these stages a list of biological processes subject to therapeutic intervention, assigning weight coefficients to these biological processes, determining optimal native therapeutic agents (genes/proteins), appropriately selecting/designing therapeutic tools—non-viral DNA vectors with cloned target genes—and composing the optimal combination of DNA vectors for each therapeutic stage.

Therapeutic Stage No. 1 – Modulation of Glial Scars in CNS Regions Subjected to Pathological Insult

Therapeutic Stage No. 1 includes the following local tasks:

1. Activation of resident microglial cells and recruitment of blood-borne monocytes to the glial-scar region.
2. Proliferation of resident microglia and migration of monocytes from peripheral blood.
3. Stimulation of monocyte and microglial differentiation into activated macrophages.
4. Polarisation of macrophages toward the M2a (reparative) phenotype.
5. Opsonisation of scar-tissue components.
6. Activation of macrophage Fc receptors and complement receptors.
7. Acceleration of phagocytosis of opsonised particles.
8. Degradation of opsonised extracellular-matrix components.
9. Conversion of reactive astrocytes within the glial scar back to a normal state.
10. Transdifferentiation of reactive astrocytes within the glial scar into oligodendrocytes.

The weight coefficients and the rationale are listed in the table below:

Local task	Weight coefficient	Comment
Activation of resident microglia and recruitment of blood-borne monocytes to the glial-scar zone	~0	Recruited microglia and monocytes transform into macrophages that clear cellular debris and scar components, thereby promoting scar remodelling. This process is already active in multiple sclerosis and does not need further stimulation.
Proliferation of resident microglia and migration of monocytes from peripheral blood	~0	Increases the number of immune cells within the scar, enhances phagocytic activity, and improves clearance of damaged tissue and regeneration-inhibiting molecules. This process is already active in MS and does not require stimulation.
Stimulation of monocyte and microglial differentiation into activated macrophages	~0	Boosts phagocytic capacity; activated macrophages more efficiently remove scar components and secrete factors that support tissue repair. This process is already active in MS and does not require stimulation.

Local task	Weight coefficient	Comment
Polarisation of macrophages toward the M2a (reparative) phenotype	~0.3	M2a macrophages perform reparative functions: they secrete anti-inflammatory cytokines and growth factors, reduce inflammation, remodel the extracellular matrix, and degrade inhibitory scar molecules.
Opsonisation of scar-tissue components*	~0.2	Tags scar components for phagocytosis; opsonisation facilitates recognition and removal of cellular debris and inhibitory molecules by immune cells, accelerating clearance of the lesion.
Activation of macrophage Fc receptors and complement receptors	~0	Enhances phagocytic activity and macrophage responses; however, activating nuclear receptor PPAR γ would have a greater impact on macrophage polarisation.
Acceleration of phagocytosis of opsonised particles	~0.2	Rapid clearance of scar components reduces scar density, frees space for axonal growth, and prevents chronic inflammation that hampers regeneration.
Degradation of opsonised extracellular-matrix components	~0	Removes inhibitory matrix molecules, facilitating axonal regeneration and neural-network repair by converting the extracellular matrix into a more growth-permissive environment. This will be addressed indirectly through macrophage polarisation to the M2a phenotype.
Conversion of reactive astrocytes within the glial scar back to a normal state	~0.3	Reduces scar formation and chronic inflammation; normalised astrocytes stop producing inhibitory factors, improving conditions for neuronal regeneration and CNS functional recovery.
Transdifferentiation of reactive astrocytes within the glial scar into oligodendrocytes	~0	Converting reactive astrocytes into new oligodendrocytes is a promising but still insufficiently studied approach; it is earmarked for a separate drug-development effort.

Therapeutic Stage No. 2: Regeneration of damaged target CNS regions and remyelination of axons in those affected areas

Therapeutic Stage No. 2 includes the following Local Tasks:

1. Activation of neural stem cells (NSCs) proliferation.
2. Differentiation of NSCs.
3. Reprogramming of astrocytes into neurons in brain regions susceptible to inflammation and degradation.

4. Neuronal polarization.
5. Neuronal migration.
6. Synthesis of tubulin, assembly and stabilization of microtubules, and regulation of their dynamics.
7. Axonal and dendritic navigation.
8. Formation of synaptic connections.
9. Differentiation of glial progenitor cells into oligodendrocyte precursor cells (OPCs).
10. Differentiation of OPCs into oligodendrocytes; formation of oligodendrocytes.
11. Enhanced synthesis of myelin by oligodendrocytes.
12. Neuroprotection.

The weight coefficients and the rationale are listed in the table below:

Local Task	Weight Coefficient	Comment
Activation of Neural Stem Cells Proliferation (NSCs)	~0	Neural stem cells are constitutively present primarily only in the dentate gyrus of the hippocampus and the subventricular zone, but as the disease progresses, even in these zones, a decrease in the number of NSCs is observed. In this regard, the process of activating NSC proliferation will be indirectly addressed through the influence of so-called hub proteins or master regulators
Differentiation of NSCs	~0	Stimulation of NSC differentiation is a process in which neural stem cells acquire the morphological and functional characteristics of mature cells necessary for performing specific tasks in the nervous system. This process will be indirectly addressed through the influence of so-called hub proteins or master regulators
Reprogramming of Astrocytes into Neurons in Brain Regions Prone to Inflammation and Degradation	~0	The process of replacing lost neurons through the transdifferentiation of astrocytes into new functional neurons is a promising approach that is not sufficiently studied at the moment but holds potential in the field of neuroregeneration. It is the subject of developing a separate pharmaceutical agent
Neuronal Polarization	~0.1	Proper polarization is necessary for the integration of new neurons into existing neural networks and the restoration of normal signal transmission
Neuronal Migration	~0	The process of neuronal movement is activated by various molecules and cellular interactions that guide neurons along specific paths to their target

		areas, where they differentiate and establish synaptic connections. This process will be indirectly addressed through the influence of so-called hub proteins or master regulators
Synthesis of Tubulin, Assembly and Stabilization of Microtubules, and Regulation of Their Dynamics	~0.1	Regulation of microtubule dynamics is important for the growth and directed projection of axons during neuron regeneration. Stable microtubules allow new neurons to properly integrate into the existing neural networks and restore synaptic connections
Axonal and Dendritic Navigation	~0.1	Following the repair of the damaged neurons, these nerve cells are supposed to establish proper connections with other neurons to form functional neural networks. Processes of axonal and dendritic navigation ensure directed growth of axons and dendrites to specific target cells, which is necessary for the restoration of synaptic connections and normal transmission of nerve impulses
Formation of Synaptic Connections	~0.2	Newly formed or restored neurons after damage must establish functional synapses with other neurons to ensure effective transmission of nerve impulses. If synaptic connections are not formed properly, the new neurons cannot integrate into the existing neural networks, thus restricting the recovery of cognitive functions
Differentiation of Progenitor Glial Cells into Oligodendrocyte Precursor Cells (OPCs)	~0.15	Expands the OPC pool, providing cells capable of maturing into oligodendrocytes—critical for restoring myelin on damaged axons and enabling remyelination
Differentiation of OPCs into Oligodendrocytes, Formation of Oligodendrocytes	~0.15	Generates new oligodendrocytes to rebuild the myelin sheath, improve impulse conduction, and accelerate neuronal recovery
Enhanced Synthesis of Myelin by Oligodendrocytes	~0	Increased myelin production ensures rapid, effective remyelination; this will be achieved indirectly through expansion of the oligodendrocyte population.
Neuroprotection	~0.2	Activation of neuroprotection mechanisms helps prevent neuronal apoptosis and slows the progression of neurodegenerative changes in the brain. This allows preserving the function of existing and newly formed segments of neural networks

III. Description of Tools

At the stage of selecting tools for delivering genetic material, the possibility of using the following platforms was considered: viral vectors, DNA vectors, and mRNA platforms.

Justification for Vector Selection:

Which delivery system is better? The "battle" between proponents of viral and non-viral vectors is less a confrontation and more an evolution of two approaches that address their unique challenges. Viral vectors are effective instruments that deliver genetic material at high speed but come with potential risks, side effects, and the need for meticulous control.

Non-viral vectors, on the other hand, are flexible and safe: they can carry genetic material with minimal risks, delivering it precisely and specifically where controlled protein expression duration is required. When massive speed or systemic delivery of genetic material is not necessary, non-viral systems are optimal. Today, non-viral vectors have become significantly more effective thanks to technological "enhancements" such as liposomal transport systems, inspired by the technological leap that occurred during the COVID-19 pandemic.

The mRNA platform has also rapidly come to the forefront in drug development in recent years. However, despite all its advantages, this platform lacks effective mechanisms for ensuring tissue specificity and protection against ectopic effects.

The answer to the question "Which carrier is better?" is clear: each tool is suitable for its purpose. The flexibility and safety of non-viral vectors are ideal where targeted delivery, prolonged but limited expression period of the target protein, and minimal risks are essential. In this project, non-viral DNA vectors of the VTvaf17 and GDTT1.8NAS series fully realize their potential.

As a pharmaceutical agent meeting the criteria for solving Local Tasks and Therapeutic Implementation, a complex of non-viral DNA vectors carrying coding regions of **native human genes** was selected. Thus, the therapeutic effect is achieved by enhancing the natural functionality of the human body.

The delivery of genetic material to target cells within the medicinal product is facilitated by unique non-viral DNA vectors of the VTvaf17 and GDTT1.8NAS series, in combination with therapeutic "coding inserts." This system represents a **biomimetic mechanism** for therapeutic intervention, promoting organism regeneration and the restoration of normal functioning of its organs and systems.

Non-viral DNA vectors of the VTvaf17 and GDTT1.8NAS series comply with stringent regulatory requirements for genetic medicinal products, as outlined in the "Draft Guideline on the quality, non-clinical and clinical aspects of gene therapy medicinal products" (EMA/CAT/80183/2014, Committee for Advanced Therapies) and the "Reflection paper on design modifications of gene therapy medicinal products during development" (14 December 2011, EMA/CAT/GTWP/44236/2009, Committee for Advanced Therapies).

DNA vectors have been developed in alignment with the recommendations of FDA-2014-D-0663, ensuring no impact on cellular proliferative activity and morphology, reproductive health, or the potential transmission of antibiotic resistance genes to the environment.

Production and quality control—including safety indicators such as apyrogenicity, sterility, stability, purity, biological activity, and others—of the produced vectors are conducted in accordance with the requirements of the U.S. Pharmacopeia (USP 42 and NF 37, Chapter 1047: Gene Therapy Products), as well as the FDA guidelines FDA-2008-D-0205, *"Chemistry, Manufacturing, and Control (CMC) Information for Human Gene Therapy Investigational New Drug Applications (INDs) Guidance for Industry,"* and FDA-2015-D-3399, *"Recommendations for Microbial Vectors Used for Gene Therapy."*

The non-viral DNA vectors of the VTvaf17 and GDTT1.8NAS series provide:

1. The highest level of safety due to:
 - Absence of regulatory elements within the DNA vectors that represent nucleotide sequences from various viral genomes.
 - No integration of DNA vectors into the genome of the target cell. Non-viral DNA vectors are used for delivering genetic material into cells but, unlike most viral vectors, remain in the cell as autonomous molecules and do not integrate into chromosomes. This avoids potential negative consequences associated with integration, such as activation of oncogenes or disruption of cellular gene function.
 - Corresponding absence of risks of spontaneous oncotransformation.
 - Absence of antibiotic resistance genes, thereby eliminating the risk of developing antibiotic-resistant strains.
 - Targeted action: A specific promoter drives gene expression exclusively in the designated cells, thereby minimising ectopic effects, immune responses, and disruptions of cellular processes. Employing a dual-promoter architecture that operates on a logical "AND" principle—a tissue-specific segment combined with an inflammation-inducible segment—provides still greater precision, ensuring delivery not only to the target cell type but specifically to those target cells within the lesion that display inflammatory signatures.
 - Encapsulation of the "DNA vector–cationic liposome" complex within an external PEG shell of low immunogenicity, which helps avoid a significant immune response.
2. Maximum efficacy due to:
 - Inclusion of highly effective promoters within the vectors.
 - Minimal size of the DNA vectors, allowing them to efficiently transfect target tissue cells without toxic effects.
 - Encapsulation of DNA vectors in cationic liposomes and PEG, protecting nucleic acids from degradation by extracellular nucleases and increasing their stability in biological fluids. This transport system enables the most efficient delivery of genetic material into cells.
 - Functionalization with specialized reagent, which is conjugated to the surface of the DNA–liposome–PEG microparticles. Functionalization with specialized reagent enhances the drug's penetration across the blood-brain barrier (BBB), and ensures maximum specificity, and affinity for cellular receptors.

- Additional efficacy—independent of the DNA-vector design or the DNA + liposome + PEG nanoparticle—is achieved through concomitant physiotherapeutic and pharmacological measures, such as focused-ultrasound (FUS), pre-emptive patient blood hydration, and other adjunctive approaches.

IV. Gene Selection

The medicinal product developer, in collaboration with engaged experts and leveraging advanced artificial intelligence-based intelligent systems and neural networks, has established an initial (baseline) list of native human candidate genes capable of executing the designated therapeutic functions.

The comprehensive task of evaluating and ranking candidate genes was addressed using the multi-round Analytic Hierarchy Process (AHP) with weighted criteria. This methodology involves decomposing the task into multiple hierarchical levels, where each subtask is individually analyzed. Therapeutic agents (candidate proteins) addressing each subtask are ranked independently, culminating in an integrated list (ranking) of candidate proteins based on these assessments.

For bioinformatics analysis, a scoring system was developed to evaluate "Benefit" ranging from +100 to 0 and "Harm" ranging from 0 to -100. This system is employed for ranking both local tasks and assigning an overall integral rating ("Weighted" final score) to each candidate gene. The "Benefit" metric accounts for the efficiency of the protein's function, as encoded by each gene, on biological processes targeted at achieving the local task. The "Harm" metric considers the risks of oncogenic disease development, adverse events, hyperexpression, and ectopic expression.

The list of candidate genes/proteins, detailed analysis of selection stages, and results of the bioinformatics evaluation of candidate genes for implementing the therapeutic stages are presented in the tables included in the Appendix.

Optimal Promoters

To select optimal promoters by evaluating the efficiency/safety ratio for multiple sclerosis therapy using DNA vectors that encode target genes, a separate bioinformatic analysis was conducted.

When choosing the best promoter for each gene, it is important to consider:

- Target and non-target cells/tissues
- Specific conditions in the target expression zone (inflammatory status, etc.)

Criteria for promoter selection—the ratio of effective expression level to the risks of ectopic expression and undesirable effects; duration of expression.

The use of cell-specific promoters can significantly reduce harm associated with ectopic expression of target proteins. Specific promoters ensure restricted gene expression only in certain cell types, minimizing the likelihood of undesirable effects in other tissues and cells. The use of a dual-promoter system based on the logical "AND" principle (a tissue-specific segment + an inflammation-inducible

segment) enables even more targeted delivery—not just to the desired cell type, but specifically to those target cells located within the lesion area that exhibit signs of inflammation.

The most significant target cells for the chosen therapeutic strategy are neurons, astrocytes, microglia, macrophages, glial progenitor cells, and oligodendrocyte progenitors.

Cell-specific promoters for projection neurons and motor neurons

1. Thy1 promoter (Thymus Cell Antigen 1)

Specificity. Active in large projection neurons of the CNS—including cortical pyramidal neurons, hippocampal neurons, and spinal motor neurons. It is expressed in neurons with long axons that transmit signals over long distances; it can also be active, to a lesser extent, in certain PNS neuron subtypes and some non-neuronal cells.

Advantages. Provides high transgene expression in target neurons, making it excellent for projection and motor-neuron targeting. Its activity in mature long-axon neurons suits studies of neurotransmission, neurodegeneration, and regeneration. Owing to its broad specificity within projection neurons, Thy1 is useful for therapies requiring robust expression across multiple CNS regions involved in cognition, movement, and sensory integration.

2. CaMKII α promoter (Calcium/Calmodulin-Dependent Protein Kinase II α)

Specificity. High specificity for mature postsynaptic neurons in the hippocampus and cortex, as well as the amygdala and other limbic structures; active mainly in post-mitotic, excitable CNS neurons—especially excitatory neurons.

Advantages. Predominantly active in mature neurons, making it ideal for targeting networks that underlie learning and memory.

3. Synapsin I promoter

Specificity. Functions mainly in neurons of the central and peripheral nervous systems, including cortical, hippocampal, subcortical, brain-stem, and spinal neurons.

Advantages. Drives expression exclusively in synaptic cells, thus restricting transgene expression to neural tissue.

Cell-specific promoters for astrocytes

1. GFAP promoter (Glial Fibrillary Acidic Protein)

Specificity. Highly specific for astrocytes in the CNS.

Advantages. Enables astrocyte-targeted gene delivery to modulate glial-scar formation and reduce astrocytic reactivity, thereby lessening scar-mediated inhibition of neuroregeneration.

2. S100 β promoter

Specificity. Expressed mainly in astrocytes and oligodendroglia.

Advantages. Facilitates delivery of therapeutic genes to astrocytes, enhancing neurotrophic support and remyelination—important for restoring axonal myelin in MS.

Cell specific promoters for microglia

1. Iba1 promoter (Ionised Calcium-Binding Adapter Molecule 1)

Specificity. Highly specific for microglia in the CNS; a marker of activated microglia.

Advantages. Targets microglia to modulate their activity, reduce neuroinflammation, and stimulate phagocytosis of myelin debris, thereby supporting remyelination and neural recovery.

2. CD11b promoter (Integrin α M)

Specificity. Active in microglia, macrophages, and monocytes.

Advantages. Delivers genes to microglia and macrophages to regulate inflammatory activity, helping to limit CNS inflammation and aid recovery in MS.

Cell specific promoters for macrophages

1. CD68 promoter

Specificity. Selective for macrophages and microglia; CD68 is a marker of phagocytic cells.

Advantages. Drives gene expression in macrophages and microglia, enhancing debris clearance and tissue repair, which promotes remyelination and reduces neuroinflammation.

2. c-fms promoter (CSF1R, Colony-Stimulating Factor-1 Receptor)

Specificity. Expressed in macrophages, microglia, and other monocyte–macrophage-lineage cells.

Advantages. Allows modulation of macrophage and microglial function, influencing their differentiation and survival to regulate inflammation and stimulate remyelination.

Cell-specific promoters for glial progenitor cells and oligodendrocyte progenitors (OPCs)

1. PDGFRA promoter (Platelet-Derived Growth Factor Receptor α)

Specificity. Highly specific for OPCs, active in proliferating and migrating progenitors pre-differentiation.

Advantages. Enables targeted expression in OPCs—valuable for strategies that boost proliferation, migration, and differentiation during remyelination therapies for MS.

2. CNP promoter (2',3'-Cyclic Nucleotide-3'-Phosphodiesterase)

Specificity. Active in early oligodendrocyte differentiation stages, including OPCs and immature oligodendrocytes.

Advantages. Targets both OPCs and early oligodendrocyte precursors, aiding studies on maturation and strategies to stimulate CNS remyelination.

3. NG2 promoter (CSPG4, Chondroitin Sulfate Proteoglycan 4)

Specificity. Highly specific for glial progenitor cells, proliferating OPCs; inactive in mature oligodendrocytes.

Advantages. Allows selective transgene expression in OPCs without affecting other cells—useful for enhancing OPC proliferation and migration to repopulate demyelinated lesions.

Considered options for inflammation-activated promoters:

1. NF- κ B Promoter:

- *Specificity:* Actively functions primarily in cells with activated transcription factor NF- κ B. This includes cells involved in immune and inflammatory responses, such as activated immune cells (macrophages, lymphocytes), vascular endothelial cells, and cells of damaged or inflamed tissues. The NF- κ B promoter is activated in response to various stimuli, including cytokines (e.g., TNF- α , IL-1 β), stress factors, infections, and tissue damage.
- *Advantages:* Expression under the control of this promoter occurs predominantly in cells with activated NF- κ B signaling, providing targeted gene expression in inflamed or damaged tissues. This allows the therapeutic genes to act only in pathological areas, reducing the risk of affecting healthy cells and minimizing side effects.

2. AP-1 (c-Fos/c-Jun) Promoter:

- *Specificity:* Actively functions mainly in cells with activated transcription factor AP-1, a heterodimer of proteins from the c-Fos and c-Jun families. The AP-1 promoter is activated in response to various external stimuli, including growth factors, cytokines, stress factors, ultraviolet radiation, and inflammatory processes. It operates in cells involved in processes such as proliferation, differentiation, apoptosis, and stress response.
- *Advantages:* Expression under the control of this promoter occurs in cells with active AP-1 signaling, providing selective gene expression in cells responding to specific stimuli or in certain physiological or pathological states. This allows targeting gene expression in cells involved in growth, development, regeneration, or responding to injury, reducing the likelihood of undesirable effects in other tissues.

3. HIF-1 α -Responsive Promoter (Hypoxia-Inducible Factor 1-alpha)

- *Specificity:* Activated under hypoxic conditions and tissue injury.
- *Advantages:* Drives gene expression only when oxygen is scarce or the tissue is damaged—situations commonly found in inflamed regions.

4. STAT3-Responsive Promoter (Signal Transducer and Activator of Transcription 3)

- *Specificity:* Triggered by immune and inflammatory signals.
- *Advantages:* Enables gene expression in direct response to inflammation, allowing precise targeting of neurons located within inflamed zones.

5. CREB-Responsive Promoter (cAMP Response Element-Binding Protein)

- **Specificity:** Activated by increased intracellular cAMP and other signalling pathways linked to neuronal activity and cellular stress.
- **Advantages:** Permits gene expression in neurons that are actively firing or under stress—conditions characteristic of damaged neural tissue.

To develop a gene therapy drug aimed at the modulation of glial scars in damaged CNS regions and neuroregeneration of damaged neurons in multiple sclerosis, it is necessary to ensure specific expression of target genes precisely in the affected cells. This can be achieved using a dual promoter that is activated only when two conditions are simultaneously met (logical "AND"): neuron specificity and activity in neurons with pathological changes.

As a result, the dual promoter will have the following construct:

1. **Neuron-Specific Part:** For example, the Synapsin I or CaMKII α promoters.
2. **Inflammation-Responsive Part:** Options include the NF- κ B response element (4–6 tandem repeats) or AP-1 (c-Fos/c-Jun).

Architecture: A "composite" promoter where regions for NF- κ B and neuronal transcription factors are adjacent, controlling a single minimal promoter upstream of the gene.

Outcome: Expression occurs only in the simultaneous presence of neuron-specific transcription factors and inflammatory transcription factors (e.g., NF- κ B). This ensures the logical "AND" function.

Thus, for gene therapy constructs intended for transfection of target cells in multiple sclerosis, the following variants of promoters have been selected:

Target cells	Promoter (with regulatory element, if any)
Neurons (neuroregeneration)	***
Neurons (protection from opsonisation)	***
Astrocytes	***
Microglia	***
Macrophages	***
OPCs, progenitor glial cells	***

Selection of Reagent for Functionalization

Functionalizing gene therapy constructs with specific ligands or antibodies allows for targeted delivery of therapeutic genes directly into target cells. This enhances the effectiveness of MS therapy through:

- Enhanced Targeted Delivery: Specific binding to target cells.
- Reduced Side Effects: Minimization of impact on unaffected tissues.
- Improved Therapeutic Response: Increased intracellular DNA delivery and expression of therapeutic genes.

Reagent options considered for functionalization for

1. Neurons:

- Peptide Angiopep-2:

Description: Angiopep-2 is a 19-amino-acid peptide derived from the low-density lipoprotein receptor-related protein 1 (LRP1). It specifically binds to LRP1 receptors expressed on endothelial cells of the blood-brain barrier (BBB) as well as on cells of the central nervous system, including neurons and astrocytes.

Advantages: Angiopep-2 facilitates efficient crossing of the BBB and ensures targeted delivery of genetic material to neurons. This leads to increased expression levels of therapeutic genes in these cells, promoting disposal of pathological intracellular protein aggregates and neuroregeneration. The use of Angiopep-2 enhances the effectiveness of MS therapy by improving drug delivery to target cells and reducing side effects associated with non-selective distribution.

- Tetanus Toxin C Fragment (TTC):

Description: TTC has a high affinity for gangliosides on the surface of neurons.

Advantages: Used for specific delivery to neurons and transport along the axonal tract.

- Antibodies Against Neuronal Markers:

Description: Antibodies targeting neuronal cell adhesion molecule (NCAM) or other neuron-specific antigens.

Advantages: Provide high-specificity binding to neurons.

2. Astrocytes

- Anti-GLAST (EAAT1) antibodies

Description: Antibodies against the astrocyte-specific glutamate transporter GLAST.

Advantages: Enable astrocyte targeting by binding surface transporters.

- L-cystine-functionalised liposomes

Description: Liposomes decorated with L-cystine, which is avidly taken up by astrocytes via dedicated transporters.

Advantages: Enhance astrocytic uptake of liposomes.

3. Microglia

- Mannosylated liposomes

Description: Liposomes modified with mannose, recognised by mannose receptors on microglia.

Advantages: Promote microglial uptake through receptor-mediated endocytosis.

- Anti-TREM2 antibodies

Description: Antibodies against the Triggering Receptor Expressed on Myeloid cells 2 (TREM2) on microglia.

Advantages: Allow specific targeting of activated microglia.

4. Macrophages

- Folate-modified liposomes

Description: Liposomes functionalised with folic acid, which binds folate receptors on macrophages.

Advantages: Increase macrophage uptake via folate-mediated endocytosis.

- Annexin V

Description: A protein that binds phosphatidylserine exposed on the surface of activated macrophages.

Advantages: Directs carriers to macrophages at inflamed sites.

- Anti-CD163 or anti-CD206 antibodies

Description: Antibodies against markers of alternatively activated (M2) macrophages.

Advantages: Provide selective delivery to M2 macrophages involved in regeneration and anti-inflammatory activity.

5. OPCs and oligodendrocytes

- Anti-LINGO-1 antibodies (Leucine-Rich Repeat and Ig-Domain-Containing Nogo-Receptor-Interacting Protein 1)

Description: LINGO-1 is expressed on oligodendrocyte surfaces and regulates their differentiation and myelination.

Advantages: Targeting LINGO-1 can foster oligodendrocyte recovery and remyelination by delivering therapeutic genes specifically.

- Peptides or antibodies against CNPase (2',3'-Cyclic-Nucleotide 3'-Phosphodiesterase)

Description: CNPase is an oligodendrocyte marker involved in myelination.

Advantages: Provides selective delivery to oligodendrocytes, enhancing axonal myelination processes.

Based on the screening results, *** has been selected for surface functionalisation. The suitability of this ligand will be validated in pilot animal studies.

Results of Bioinformatic Analysis of Target Genes/Proteins

Based on the processing results using a three-round Analytical Hierarchy Process methodology, and with due consideration of the weight coefficients of the Local Tasks, the following genes/proteins have been included in the final (key) list:

Therapeutic Stage No. 1:

Gene / protein	Local task	Rationale for inclusion	Coding region (bp)	Protein size (kDa)	Secreted	Cellular localisation
***	***	***	***	***	***	***
***	***	***	***	***	***	***
***	***	***	***	***	***	***
***	***	***	***	***	***	***
***	***	***	***	***	***	***
***	***	***	***	***	***	***

In MS therapy, enhancing opsonisation of the glial scar—and thus increasing complement-receptor activity on phagocytic cells—is highly desirable: it promotes removal of astroglial scar tissue and improves access to the lesion for subsequent remyelination. The same mechanism, however, risks opsonising and phagocytosing synapses on intact or partly damaged yet still functionally valuable neurons, an unresolved dilemma in conventional pharmacology (the “complement dilemma”).

In the present drug design, the complement dilemma is addressed as follows: to prevent unwanted clearance of synapses, neurons are transfected with a gene encoding ***, the canonical “don’t-eat-me” signal. Its expression ensures that, even in the presence of complement tags, synaptic phagocytosis is selectively blocked.

Therapeutic Stage № 2:

Gene / protein	Local task	Rationale for inclusion	Coding region (bp)	Protein size (kDa)	Secreted	Cellular localisation
***	***	***	***	***	***	***
***	***	***	***	***	***	***
***	***	***	***	***	***	***
***	***	***	***	***	***	***
***	***	***	***	***	***	***
***	***	***	***	***	***	***

To ensure controlled interactions, the composition of the pharmaceutical formulation may include no more than five genes for each Therapeutic Stage. The final selection will be determined through in vivo experiments.

Achieving the therapeutic effect is ensured by the multiplicative impact resulting from the inclusion of genes that regulate biological processes across all Therapeutic Stages in the pharmaceutical composition. Additionally, the risks of ectopic gene expression included in the composition have been considered and minimized by selecting optimal promoters.

The technical implementation of this concept involves the creation of therapeutic non-viral DNA vectors based on the VTvaf17 and GDTT1.8NAS platform solution for each protein/gene included in the final (key) list. The list of therapeutic DNA vectors is provided in the table below:

DNA vector	Therapeutic gene	Promoter	Total size (bp)
***	***	***	***
***	***	***	***
***	***	***	***
***	***	***	***
***	***	***	***
***	***	***	***
***	***	***	***

DNA vector	Therapeutic gene	Promoter	Total size (bp)
***	***	***	***
***	***	***	***
***	***	***	***
***	***	***	***
***	***	***	***

Finished Dosage Form (FDF)

Criteria for Selecting FDF (in descending order of importance):

- Therapeutic Efficacy: Minimization of adverse effects.
- Ensuring Maximum Preservation of Drug Functionality.
- Ease of Application, Transportation, and Storage.
- Affordable Cost Characteristics.

Proposed FDF Options:

1. Lyophilized DNA Vector Complex for Solution Preparation: DNA vectors without a coating are rapidly cleared in the cerebrospinal fluid and blood, which does not meet the criterion for preservation.
2. Lyophilized DNA Vector Complex Encapsulated in Liposomes and Polyethylene Glycol (PEG) for Solution Preparation: Double encapsulation requires a more thorough assessment of additional toxic effects. Additionally, this option may result in a slight increase in production costs.
3. Lyophilized DNA Vector Complex Encapsulated in Liposomes and Polyethylene Glycol (PEG) with Functionalization with specialized reagent for Solution Preparation: specialized reagent is conjugated to the surface of DNA vector + liposome + PEG microparticles. Functionalization with specialized reagent enhances drug penetration through the blood-brain barrier (BBB), specificity, and affinity to cellular receptors.
4. Lyophilized DNA Vector Complex with PEIpro Transfection Reagent (Polyethyleneimine) for Solution Preparation: Using polyethyleneimine as a transport system may cause side effects and undesirable phenomena.
5. Lyophilized DNA Vector Complex Encapsulated in Cationic Liposomes for Solution Preparation: Ensures maximum efficacy due to a high level of target cell transfection and preserves DNA vectors through encapsulation in cationic liposomes, with minimal adverse effects.

Final Selection:

The chosen final pharmaceutical form is a lyophilized DNA vector complex with double encapsulation—encapsulated in cationic liposomes and PEG with functionalization for infusion solution preparation. Encapsulation of DNA vectors with cationic liposomes in polyethylene glycol (PEG) can significantly enhance the efficiency of drug penetration into the target brain regions. PEG provides improved biocompatibility, prolongs the circulation time of liposomes in the cerebrospinal fluid and bloodstream, and reduces drug recognition by the immune system, thereby protecting it from degradation. Specialized reagent for functionalization enhances drug penetration through the BBB, specificity, and affinity to cellular receptors.

This type of FDF can substantially increase the efficiency of drug penetration into the target brain cells compared to the baseline level (DNA + cationic liposome).

V. Delivery Methods

In multiple sclerosis pathological changes follow characteristic patterns that primarily involve the central nervous system (CNS) and, in some cases, extend to the peripheral nervous system (PNS). After ranking the affected regions by clinical importance, the following priorities emerge:

CNS — very high priority.

- White-matter tracts of the brain, especially the periventricular regions, corpus callosum, cerebellum, and brain-stem pathways, are the most vulnerable. Demyelination here disrupts motor control, coordination, and cognition.
- Cervical and thoracic segments of the spinal cord are also frequently involved; lesions at these levels produce notable motor deficits, sensory loss, and autonomic problems such as bladder and bowel dysfunction.

CNS — high priority.

- The cerebellum, responsible for balance and fine motor coordination, when damaged leads to ataxia and tremor.
- The optic nerve; optic neuritis is a common early sign of MS. Demyelination in this nerve causes reduced visual acuity, pain on eye movement, and transient vision loss.

CNS — moderate priority.

- Subcortical nuclei can be affected, with consequences for muscle tone, cognition, and emotional regulation.
- Cortical areas are less often involved, but lesions here may still give rise to cognitive and speech disturbances.

Moving to the peripheral nervous system:

PNS — high priority.

- Spinal nerve roots may be secondarily affected owing to central lesions, leading to radicular pain and paraesthesias.

PNS — moderate priority.

- Peripheral nerves themselves are only rarely damaged directly in MS, yet systemic inflammatory mediators can occasionally produce peripheral-nerve symptoms.

Method of administration

Reaching the deep layers of the brain regions affected by pathological processes in MS with a drug is one of the most serious challenges and tasks of this project.

The method of administration should provide a significant therapeutic level of drug distribution in the target brain regions and create optimal conditions for effective transfection of target cells, with subsequent expression of therapeutic proteins.

The considered methods of drug administration are: intrathecal, intra-arterial. The choice of the method of drug administration should also take into account the criterion of drug preservation (DNA encapsulated in protective sheaths) for maximum prolonged presence/circulation in the medium in the area of administration and readiness for transfection of target cells.

Intrathecal Administration

When a pharmaceutical composition comprising DNA vectors encoding target proteins, combined with liposomes, is administered intrathecally (into the lumbar region of the spinal canal), it directly enters the cerebrospinal fluid (CSF). This method allows bypassing the blood-brain barrier (BBB) and provides direct access of the drug to the central nervous system (CNS).

1. Distribution of the Drug in the Cerebrospinal Fluid:
 - The cerebrospinal fluid is produced in the choroid plexuses of the brain's ventricles and circulates through the ventricles and the subarachnoid space of the brain and spinal cord.
 - Following intrathecal administration in the lumbar region, the drug ascends with the flow of CSF through the subarachnoid space of the spinal cord toward the brain.
2. Overcoming Barriers Between CSF and Brain Tissue:
 - The outer surface of the brain is covered by the pia mater and a thin layer of astrocytic processes (the glial limiting membrane).
 - The pharmaceutical penetrates this barrier via paracellular diffusion or through transport mechanisms such as transcytosis across endothelial cells.
 - The DNA, encapsulated in protective shells, utilizes perivascular spaces (Virchow-Robin spaces) surrounding blood vessels to infiltrate the deeper layers of brain tissue.
3. Interaction with Brain Cells:
 - After penetrating the brain parenchyma, the liposomal DNA vectors are in close proximity to neurons. Cationic liposomes interact with the negatively charged cell membrane, stimulating uptake via endocytosis.

4. Release of DNA Vectors Inside Cells:

- Once inside endosomes, the liposomes disrupt the endosomal membrane, releasing the DNA vectors into the cytoplasm. The liposomal coating protects the DNA from intracellular nucleases until release.

5. Transport of DNA to the Nucleus and Gene Expression:

- A limited number of DNA vectors enter the nucleus of the transfected cell. Within the nucleus, mRNA synthesis occurs from the DNA vector, and target proteins are synthesized on ribosomes in the cytoplasm.
- The dual promoter ensures gene expression in target cells exhibiting an inflammatory status, minimizing ectopic expression in other cell types.

Arterial Infusion:

1. Preventive Administration of Mannitol and Intravenous Hydration

- Mannitol: As an osmotic diuretic, mannitol temporarily increases plasma blood osmolarity. Elevated osmolarity leads to dehydration of endothelial cells in cerebral capillaries, causing their volume to shrink. The reduction in endothelial cell volume creates temporary intercellular gaps, enhancing the permeability of the Blood-Brain Barrier (BBB).
- Intravenous Hydration: Nucleases are enzymes that degrade nucleic acids, including DNA. Increasing the fluid volume in the bloodstream through intravenous hydration dilutes nucleases. Reduced nuclease concentration decreases the likelihood of DNA complexes being degraded before reaching target cells. Additionally, hydration reduces blood viscosity, improving blood flow and promoting a more uniform distribution of DNA+liposome+PEG particles throughout the vascular system, thereby enhancing their access to areas with temporarily compromised BBB integrity.
- Maintaining Osmotic Balance: Hydration helps sustain osmotic equilibrium, preventing osmotic stress that could damage liposomal nanoparticles and the DNA vectors encapsulated within them.
- Compensation for Mannitol's Osmotic Effect: Mannitol, as an osmotic diuretic, may cause fluid loss from cells and tissues. Intravenous hydration compensates for this effect, maintaining overall fluid volume within the body.

2. Ultrasound Stimulation with Microbubbles

- Targeted Focused Ultrasound (FUS): Utilizing FUS enhances the efficiency of drug penetration into target brain regions and significantly improves the delivery of therapeutic agents. When combined with liposomes, FUS increases the permeability of brain tissues and temporarily opens the BBB.
 - Ultrasound Impact: Exposure to ultrasound causes microcavitation of microbubbles near endothelial cells.
 - Mechanical Disruption: The mechanical effects of ultrasound can temporarily disrupt the tight contacts between endothelial cells, increasing the permeability of the BBB.

- Precision Targeting: Focused ultrasound allows you to target specific areas of the brain.
3. Intraarterial Drug Administration
 - Ensures rapid attainment of high drug concentrations within the arterial system supplying the brain. Administering the drug directly into an artery leading to the brain enhances its efficacy and reduces systemic exposure to other organs.
 4. Overcoming the Blood-Brain Barrier (BBB)
 - Mannitol and ultrasound stimulation jointly provide a temporary, reversible, and controlled reduction of the BBB's barrier function. The formation of intercellular gaps and the expansion of existing pores allow macromolecules and nanoparticles to penetrate the brain parenchyma. Additionally, functionalizing microspheres with specialized reagent further enhances drug penetration through the BBB.
 5. Transport of DNA+Liposome+PEG Complexes to Brain Tissue
 - DNA+liposome+PEG complexes have an average size of about 150 nm: The size allows them to pass through the enlarged pores in the BBB. Polyethylene glycol (PEG) provides increased circulation time in the bloodstream and decreased uptake by the reticuloendothelial system.
 - Circulation in the Bloodstream:
 - The first circulation: Maximum concentration of "DNA-liposome-PEG" complexes reaches brain capillaries immediately after administration.
 - Passive Penetration: Complexes diffuse into the brain interstitial space through temporarily opened BBB gaps.
 6. Interaction with Brain Cells
 - Upon penetrating the brain parenchyma, liposomal DNA vectors are in close proximity to neurons. Cationic liposomes interact with the negatively charged cell membranes, promoting uptake via endocytosis. Functionalizing microspheres with specialized reagent further enhances specificity and affinity for cellular receptors.
 7. Release of DNA Vectors Inside Cells
 - Once inside endosomes, liposomes disrupt the endosomal membrane, releasing DNA vectors into the cytoplasm. The liposomal shell protects DNA from intracellular nucleases until release.
 8. Transport of DNA to the Nucleus and Gene Expression
 - A limited number of DNA vectors enter the nucleus of transfected cells. Within the nucleus, mRNA synthesis from the DNA vector occurs, and target proteins are synthesized on ribosomes in the cytoplasm.
 - Dual Promoter System ensures gene expression in target cells exhibiting inflammatory status, minimizing ectopic expression in other cell types.

At the current project stage, a comprehensive analysis of the outlined administration methods was conducted based on the criterion of maximum drug delivery to target brain regions. The following methods were selected: intrathecal administration, arterial infusion with preventive mannitol administration, intravenous hydration, and focused ultrasound stimulation (FUS).

The selected administration methods ensure a substantial therapeutic level of drug distribution within the target brain regions and create optimal conditions for the effective transfection of target cells, with subsequent expression of therapeutic proteins.

Priority Administration Method: Intraarterial infusion with preventive administration of mannitol, intravenous hydration, and FUS has been identified as the priority method. The final decision regarding the most effective administration route will be made based on the results of animal experimentation.

VI. Treatment Protocols

The flexibility of therapeutic regimens is ensured by tools for adapting the schedule/scheme of drug administration with due regard to the effect and adverse events:

- Duration: The therapeutic agent exhibits a prolonged (~20 days) yet observable period of action (expression of therapeutic genes) in the target regions of the brain.
- Frequency: The therapeutic agent allows for multiple administrations in the absence of immunogenicity.
- Dosage: Personalized therapeutic scenarios can be developed based on actual efficacy and adverse events.

The administration of the therapeutic agent can be organized according to various protocols depending on the disease characteristics, therapeutic objectives, and the pharmacokinetics of the drug. The main administration schemes are outlined below:

Single Administration

- Description: The drug is administered once to achieve an immediate therapeutic effect. It is used when a quick intervention is needed to treat a disease or symptom.
- Advantages: Rapid action, simplicity.
- Disadvantages: Potential for a transient effect, high initial concentration may cause side effects.

Revolver Administration with Cumulative Effect

- Description: The drug is administered multiple times with gradual accumulation in the body until therapeutic concentration is achieved. The cumulative effect is ensured by the drug's prolonged half-life or its accumulation in tissues.
- Advantages: Achieves a stable therapeutic effect during prolonged treatment. Maintains an even concentration of the therapeutic agent in the body.
- Disadvantages: Risk of accumulation and toxicity, necessity for monitoring drug concentration.

Revolver Administration with Complete Reduction of Therapeutic Effect (Intermittent)

- Description: The drug is administered multiple times, but the therapeutic effect diminishes between administrations. This may be due to the drug's rapid metabolism and elimination. Restoration of the therapeutic effect requires subsequent administrations.
- Advantages: Controlled impact on the body with the ability to adjust dosages.
- Disadvantages: Risk of "peak" effects and side reactions during high concentration levels of the therapeutic agent.

Pulse Administration (Pulsed Therapy)

- Description: The drug is administered in large doses at intervals to achieve a significant effect over a short period while minimizing side effects.
- Advantages: Rapid symptom relief, reduced side effects between administrations.
- Disadvantages: Risk of adverse events at high doses.

Multi-Stage Administration

- Description: The drug is administered sequentially, with each stage targeting a specific objective (e.g., utilization of pathological intracellular α -synuclein aggregates; neuroregeneration).
- Advantages: Effective adaptation to changing treatment conditions.
- Disadvantages: Requires meticulous planning and monitoring.

Selected Treatment Scheme:

A treatment regimen was selected to maximize drug efficacy and minimize adverse events:

Multi-Stage Pulse Administration, Multi-Course (as Indicated):

Initially, each therapy course involves the pulse administration of the first stage drug, containing DNA vectors encoding genes responsible for implementing Therapeutic Stage No. 1: Modulation of glial scars in the damaged CNS areas. After a certain period (~14 days), the administration of the second stage drug occurs, containing DNA vectors encoding genes responsible for implementing Therapeutic Stage No. 2: Regeneration of Targeted Brain Regions damaged by MS, remyelination.

Course Treatment Scheme:

1. First Pulse Administration of Stage One
2. Exposure Period: 6 days
3. Second Pulse Administration of Stage One
4. Exposure Period: 6 days
5. First Pulse Administration of Stage Two
6. Exposure Period: 6 days
7. Second Pulse Administration of Stage Two
8. Exposure Period: 60 days
9. Disease Remission Control

Therapy courses may be repeated as indicated until a significant therapeutic effect is achieved.

VII. Economic Feasibility

According to external experts involved, the economic indicators for the holder of the drug's registration certificate are expected to be quite impressive, provided that preclinical and clinical studies are successfully completed and the drug subsequently enters the market.

Aspects Considered in Evaluating Economic Indicators:

- **Multifactorial Nature of the Disease:** The extraordinary complexity of multiple sclerosis and the absence, as of the date of this material, of widely accepted therapeutic solutions with confirmed efficacy and safety have led to a significant unmet demand.
- **Utilization of Established Technological Approaches:** The project employs proven technological methods, enabling the drug to be manufactured at various standard (non-unique) biotechnological facilities. This approach aims to achieve acceptable pricing while ensuring high profit margins for the holder of the drug's registration certificate.
- **Convenient Logistics and Storage Conditions:** The developed pharmaceutical— a lyophilized complex of DNA–cationic liposomes–PEG—offers ease of logistics and storage, facilitating widespread use in various countries.
- **The rising number of patients—and the improved early detection rate of multiple sclerosis—**are creating the conditions for a rapidly expanding, massive market segment whose demand is currently unmet.
- **Scope of Preclinical and Clinical Studies:** Due to the extensive nature of the required preclinical and clinical studies, which necessitate the involvement of a global player, the implementation envisions the creation of an alliance or consortium for further advancement of the project.

Accordingly, project participants using the following approaches in the development of drugs namely:

- modern nature-like molecular genetic tools (unique non-viral DNA vectors);
- complex bioinformatics algorithms for realization of therapeutic stages (evaluation and ranking of candidate genes/proteins by methods of multi-round analytical hierarchy, delivery methods, finished dosage form composition, etc.);
- combinatorics (optimal combination of genes/proteins responsible for disposal of pathological intracellular protein aggregates, regeneration of target brain sections; the use of tools for specific delivery of the medication to target cells (promoters, reagents for functionalization); optimization of the finished dosage form composition, determination of the method of administration; application of additional physiotherapeutic methods in therapy),

taking into account the indicated market factors have increased chances of successful launch of the drug into the market and its commercialization.

It should also be considered that the indications for the use of the developed pharmaceutical may, in the future, be expanded to other similar conditions.

Completed Steps

1. Bioinformational Elaboration: Identification of Base List of Candidate Genes/Proteins

Utilizing extensive bioinformatics research conducted by both engaged molecular biology experts and artificial intelligence, an initial (base) list of candidate genes/proteins was established. This list includes genes/proteins responsible for the utilization of pathological intracellular protein aggregates and the regeneration of targeted brain regions.

2. Bioinformational Elaboration: Ranking and Formation of Key List of Candidate DNA Vectors

The genes/proteins included in the initial (base) candidate list were ranked using the Analytic Hierarchy Process with Weighted Criteria (AHP). This method involves breaking down the task into multiple levels, where each local task is analyzed separately. Therapeutic tools (candidate proteins) for solving local tasks are ranked individually, and an integrated list (ranking) of candidate proteins is subsequently formed. As a result, a key (condensed) list of candidate genes/proteins was made up.

3. Development of coding cassettes for each vector candidate and local therapeutic task for subsequent incorporation into DNA vectors.

Identification of optimal cell type–specific promoter elements, inflammation-inducible regulatory promoter regions, and accordingly the optimal dual-promoter configuration. Construction of promoter segments for each vector candidate and local therapeutic task for subsequent incorporation into DNA vectors.

4. Creation of DNA Vectors

Platform DNA vectors of the VTvaf17 and GDTT1.8NAS series were developed.

5. Depositing Production Strains

Original producer strains for the industrial production of the specified vectors were deposited in international repositories such as NCIMB and others.

6. Intellectual Property Registration

All necessary patent procedures were completed to register intellectual property rights: patents were filed for inventions related to the VTvaf17 and GDTT1.8NAS platform DNA vectors and the corresponding producer strains. List of patents: US11149279, EP3673061, CN111065738, JP6918231, CA3073258, KR102276373, IL272823, US20210310011, US2022000855, EP3847252, CN113166771.

7. Production and Research

Preliminary in vitro preclinical studies of the platform DNA vectors were conducted. Production technology was developed, and laboratory and pilot-industrial manufacturing protocols were established. Preclinical studies and Phase I clinical trials of another drug (for a different indication) have been conducted using the same platform DNA vectors.

8. Development of the Finished Dosage Form (FDF)

The transport system selected was cationic liposomes. In vitro and in vivo experiments were conducted to evaluate the transfection/expression efficiency of the target gene by the DNA-liposome complex. The preferred variant of Finished Dosage Form in the form of DNA-cationic liposome-PEG with functionalization was developed. Ligands for nanoparticle functionalization have been identified. The optimal size of the DNA + liposome + PEG nanospheres has been determined. Production technology options for the Finished Dosage Form were developed.

9. Determination of Administration Method

A comparative evaluation of administration methods was performed: intra-arterial and intrathecal. The preferred administration method was identified as intra-arterial. Additional pharmacological (mannitol, hydration) and physiotherapeutic (Focused Ultrasound, FUS) methods were selected to ensure maximum penetration of the therapeutic agent into the patient's target brain tissues.

10. Development of Preclinical Trials Plan

A comprehensive preclinical trials plan was developed to assess the efficacy and safety of each candidate gene/protein and to determine the final composition of DNA vectors within the drug formulation:

Selection of animal model:

The most widely used model of multiple sclerosis is experimental autoimmune encephalomyelitis (EAE), in which animals are immunised with peptides derived from PLP (proteolipid protein) or MBP (myelin basic protein). By varying the dosing schedule, one can reproduce either the relapsing-remitting or the chronic form of the disease. The model has several features that must be taken into account. Lesions develop in a rather random pattern, which makes precision assessment of remyelination difficult. SJL/J mice should be used, as they are naturally prone to demyelination and highly susceptible to EAE induction (notably, females are far more sensitive than males).

Three-stage experimental plan

1. Stage 1 – Glial-scar modulation

EAE is induced, preferably in its chronic form, because axonal damage and myelin loss are then more extensive. By day 20 after immunisation with the MOG35-55 peptide, the animals should show clear motor deficits on the standard clinical score. At that point the first therapeutic cocktail is administered. One month later:

- Motor performance is assessed.
- NF200 immunostaining is used to detect damaged axons.
- The degree of myelination is evaluated with MBP staining.
- Microglial activation is assessed with the Iba1 marker, astrocyte numbers with GFAP.
- Polarised M2 macrophages are quantified by immunostaining for c-Myc.
- Laser confocal microscopy is used to count dendritic spines.

- PCR and Western blot analyses measure expression of pro-inflammatory cytokines, complement proteins, and CSPGs.

2. Stage 2 – Neuroregeneration and remyelination

EAE is induced again, but the second therapeutic cocktail is administered. One month later:

- Motor functions and baseline cognition are measured (recency test for short-term memory, novel-object-recognition for long-term memory).
- Co-localisation of *** with the ER marker calreticulin is analysed to gauge CDNF secretion.
- Myelination is assessed with MBP staining.
- Mature oligodendrocytes are counted by APC immunostaining.
- Dendritic length, branching, and spine numbers are quantified on brain sections.

3. Stage 3 – Combined treatment

EAE is induced; the Stage 1 cocktail is administered, and one month later the Stage 2 cocktail. Motor and cognitive tests are performed one month after the second injection. Brain samples are then examined for all morphological and molecular markers listed above—synaptic and cytoskeletal integrity, myelination, microglial and astrocytic responses—by immunohistochemistry and Western blot. These data will provide a comprehensive picture of the efficacy of the selected gene combinations and help identify the optimal subset of therapeutic proteins.

Upcoming Steps

- Creation of therapeutic DNA vectors - candidates carrying target genes and the corresponding producer strains. Preliminary work on the development (design) of DNA vectors is underway.
- Conducting a series of experiments on animals (to determine DNA vectors included in the final composition of the drug; verification of the method of drug administration),
- Following the results of animal experiments: determination of the final composition, DNA vectors ratio, DNA/liposome/PEG ratio in the drug product. Preparation of the final Finished Dosage Form,
- Transfer of DNA vector and the Finished Dosage Form manufacturing technology to an industrial pharmaceutical company,
- Conducting a set of regulated preclinical studies of the drug to assess efficacy / safety,
- Conducting a set of clinical trials of the drug to assess efficacy / safety, and in case of successful implementation of these stages
- Registration and placing the drug on the market
- Expansion of the indications for use and the number of patients covered