Genoterosil: Gene Therapy for Bone Tissue Regeneration

Project Concept for the Development of the Genoterosil Gene Therapy Aimed at Accelerating Bone Regeneration

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 Biological Processes**. Priority is given to selecting therapeutic targets that directly impact the biological processes underlying bone tissue regeneration and fracture healing, such as activation of osteogenic signaling pathways and formation of the bone matrix.
2. **Multiplicity of Targets**. Therapeutic versatility is achieved through the use of a composition of next-generation DNA vectors carrying multiple genes designed to act upon relevant therapeutic targets.
3. **Unique DNA Vectors**. Our therapeutic DNA vectors of VTvaf17 series developed and patented as a platform solution, elegantly combine efficacy, safety, and the flexibility to vary with promoters and coding sequences of target genes.
4. **Precision Expression**. By employing carefully selected promoters and administering the product under specialized imaging guidance, we ensure efficient expression of target genes, optimize targeted drug delivery to the fracture site and minimize risks to the patient. This strategy enhances overall therapeutic efficacy while reducing potential side effects.
5. **Optimal Delivery System**. Using a calcium-phosphate precipitate as the carrier system ensures both high efficiency and safety in delivering genetic material to cells. This carrier further enhances bone regeneration by promoting mineralization of the bone matrix.
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 bone tissue regeneration 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.

Key Components and Development Stages of the Genoterosil:

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

In designing a robust strategy, we propose using only validated approaches, data, and arguments, i.e., to use experimental methods to confirm the correlation between dynamic shifts in biological indicators and changes in the expression levels of target genes.

Osteogenesis is a complex, multi-stage sequence of events that leads to the formation and restoration of bone tissue. Early on, the inflammatory response plays a pivotal role as a "danger signal," mobilizing immune cells and preparing a favorable environment for subsequent processes. As the inflammation subsides, a variety of cellular and molecular mechanisms come into play: mesenchymal stem cells, growth factors (BMP, TGF-β, VEGF, etc.), osteoblasts, osteoclasts, and endothelial cells that form new blood vessels. The interplay and coordinated activity of these elements result in the synthesis and mineralization of the bone matrix, followed by further tissue remodeling. Ultimately, this orchestrated process produces mature, fully functional bone.

The project strategy also covers conditions in which physiological bone repair is inherently weak or pathologically altered—including osteogenesis imperfecta, skeletal dysplasias, osteoporosis, non- and delayed-union fractures, and pseudarthrosis. Under normal circumstances, when the fracture site has an adequate level of osteogenic stimuli and synthesizes enough structural proteins to form the bone matrix, the healing follows the classic pathway: hematoma → inflammation and debridement → soft (cartilaginous) callus → hard (bony) callus → remodeling into mature bone. If at any point regeneration is disrupted or follows a pathological course (e.g., due to inadequate fracture stabilization, insufficient production of growth factors, damaged blood supply), the defect area becomes occupied by fibrous or cartilaginous tissue that does not develop into a fully mineralized bony callus.

Genoterosil involves mitigation of the risks of pseudarthrosis and disability through several mechanisms. Firstly, it promotes enhanced osteoinduction: the addition of exogenous osteogenic signals accelerates the formation of a robust bone matrix, causing osteoblasts and mesenchymal stem cells to intensify bone tissue synthesis rather than fibrous tissue. Secondly, the drug augments the production of structural proteins: a high local concentration of collagenous components provides a "scaffold" that fortifies the bone and prevents its conversion into fibrous tissue. Thirdly, it improves the mechanical stability of the bone callus: when the bone matrix develops more rapidly and with greater quality, the fracture gains earlier primary stability, reducing micromotion and consequently the likelihood of pseudarthrosis. Finally, the product acts synergistically with conventional fracture stabilization methods (splints, plates, screws, etc.): the additional delivery of osteogenic factors and structural proteins fosters optimal conditions for transforming fibrous or cartilaginous callus into bone, thus substantially lowering the risk of forming a false joint, which often leads to chronic pain, restricted mobility, and ultimately disability.

By ensuring a high concentration of pivotal regulators and bone matrix components, Genoterosil facilitates the timely initiation and proper progression of all stages of osteogenesis. As a result, it not only significantly shortens the time to fracture union and the patient's duration of incapacity but also markedly reduces the chance of a pathological "stall" in the fibrous-tissue phase, thereby minimizing the risk of pseudarthrosis and subsequent disability.

The project strategy envisions creating Genoterosil to therapeutically implement biological processes directly linked to the underlying condition. A detailed rationale for the inclusion of these biological processes and priority therapeutic objectives in the project strategy is presented in the following table:

Biological Processes / Pathogenesis	Therapeutic Objective	Inclusion in Project Strategy (Yes/No)	Comment
Inflammation	Optimization of inflammatory processes	No	There is no direct objective in the current project to suppress excessive inflammation; a moderate inflammatory response is a native (physiological) reaction to injury and is beneficial for initiating primary regenerative and reparative processes. In cases of overly active inflammatory processes, existing, effective market therapies can be utilized.
Early Bone Morphogenesis	Activation of osteogenic signaling pathways by increasing the expression of bone growth factors	Yes	Bone growth factors are key to the rapid initiation and activation of osteogenesis; local expression of these factors activates osteoblasts and mesenchymal stem cells, triggering the formation of new bone. Achieving high-quality early bone morphogenesis is a prerequisite for developing fully functional bone at the fracture site and preventing complications (e.g., pseudarthrosis).
Formation of the Bone Matrix	Accelerated formation of the bone matrix via enhanced expression of structural proteins	Yes	The formation of the bone matrix is fundamental to creating fully functional bone at the fracture site and represents a crucial therapeutic stage. High-quality execution of this process is essential for preventing complications such as pseudarthrosis.
Mineralization	Acceleration of bone matrix mineralization via bone growth	Yes	Mineralization of the bone matrix is critical for successful bone regeneration, first forming primary (woven) bone and subsequently more structured lamellar bone. By

Biological Processes / Pathogenesis	Therapeutic Objective	Inclusion in Project Strategy (Yes/No)	Comment
	factors and creation of a calcium-phosphate reserve in the fracture area		incorporating calcium phosphates, hydroxyapatite crystals form more intensively within the collagenous scaffold, thereby enhancing the mechanical strength of the new bone tissue.
Formation of Primary Bone Tissue	Improved development of a firm bone callus and primary bone tissue through coordinated action of additional growth factors and structural proteins	Yes	The formation of primary bone tissue is a key therapeutic objective. This stage is foundational for generating fully functional bone at the fracture site and preventing complications such as pseudarthrosis.
Formation of Organized Lamellar Bone	Enhanced formation of lamellar, mature bone tissue through coordinated action of additional growth factors and structural proteins	Yes	The creation of mature lamellar bone is a relatively prolonged phase and the final therapeutic objective. Achieving full-fledged functional bone at the fracture site prevents complications such as pseudarthrosis and ensures complete patient recovery.
Angiogenesis	Stimulation of angiogenesis to improve blood supply and metabolism in the fracture zone	Yes (indirectly)	Vascular ingrowth is partly improved when bone growth factor levels increase, though no direct emphasis on angiogenesis has been included in the project strategy. Nevertheless, this aspect can be indirectly addressed by other factors promoting vascularization.

II. Therapeutic Implementation

The therapeutic implementation ("Integral Task") employed in this project comprises:

- Defining the list of biological processes subject to therapeutic intervention;
- Identifying native therapeutic genes to create a composition targeting all designated biological processes;
- Selecting/designing therapeutic tools—non-viral DNA vectors containing the cloned target genes, an optimal promoter, and other structural elements that meet the therapeutic requirements;

- Establishing the final dosage form (FDF), dosage regimen, administration route, and treatment protocols that fulfill the therapeutic objectives of the project.

In line with the project strategy, a detailed set of therapeutic objectives and corresponding biological mechanisms has been specified for targeting by the medicinal product:

1. Intensification of Early Bone Morphogenesis
    - Enhancing the proliferation and migration of mesenchymal stem cells (MSCs) to the site of injury.
    - Stimulating the differentiation of MSCs into osteoblasts and chondroblasts during the initial stages of regeneration.
    - Increasing the synthesis and secretion of extracellular matrix components, particularly collagen.
2. Acceleration of Bone Matrix Formation
    - Amplifying the production of structural proteins that form the “scaffold” of the bone matrix.
    - Boosting collagen fiber synthesis for more rapid development of the organic matrix (osteoid), which serves as the foundation for subsequent mineralization.
3. Acceleration and Intensification of Mineralization
    - Speeding the mineralization of the bone matrix by promoting the active uptake of calcium and phosphate ions by osteoblasts in newly formed tissue.
    - Providing additional impetus for hydroxyapatite crystal formation within the collagen framework, leading to faster acquisition of mechanical strength in bone tissue.
4. Enhancement of Speed and Quality in Primary Bone Tissue Formation
    - Fortifying the bone regenerate and improving the formation of the bone callus through the coordinated action of growth factors and structural proteins.
    - Expediting the transition from the fibrous-cartilaginous regeneration phase to the development of primary (woven) bone tissue.
5. Intensification of Organized Lamellar Bone Formation
    - Activating the remodeling of woven bone into mature lamellar bone.
6. Promotion of Angiogenesis and Metabolism (Indirectly via Increased Levels of Bone Growth Factors)
    - Indirect stimulation of angiogenesis, improved blood supply, and metabolism in the fracture zone, achieved through the secretion of angiogenic factors by more active osteoblasts and associated cells (endothelial cells).

- Optimizing blood supply to expedite the delivery of nutrients and the recruitment of new cells involved in bone tissue restoration.

III. Description of Tools

During the selection of methods for delivering genetic material, three potential platforms were considered: viral vectors, DNA vectors, and mRNA-based systems.

Rationale for Choosing a Vector

Which delivery system is best? The “battle” between advocates of viral and non-viral vectors is less a direct confrontation and more an evolution of two approaches, each suited to its own unique purposes. Viral vectors are powerful tools that deliver genetic material with high efficiency but come with potential risks, side effects, and the need for rigorous oversight.

Non-viral vectors, by contrast, offer flexibility and safety; they can carry genetic material with minimal risk, delivering it carefully and specifically to the cells requiring controlled-duration expression. In situations where the highest possible transfection/transduction efficiency or systemic delivery of genetic material is not required, non-viral systems are optimal. Advances in technology have significantly improved non-viral vectors, enhancing their overall performance.

While mRNA-based platforms have come to the forefront of drug development in recent years, they lack effective mechanisms for tissue specificity and for preventing off-target effects, despite their many advantages.

Consequently, the answer to the question “Which carrier is better?” depends on the specific objective. The flexibility and safety of non-viral vectors make them ideal when targeted delivery is essential, when a long but finite protein expression profile is required, and when minimal risks are paramount. In this project, non-viral DNA vectors of the VTvaf17 series fully realize this potential.

As a medicinal product aligned with the Therapeutic Implementation criteria, a complex of non-viral DNA vectors carrying native human gene sequences was chosen. This achieves a therapeutic effect by enhancing the body’s own natural functionality.

These unique non-viral VTvaf17-series DNA vectors, equipped with therapeutic “coding inserts,” serve as a biomimetic mechanism for therapeutic action, facilitating tissue regeneration and the restoration of normal organ/system function.

Regulatory Compliance

VTvaf17-series non-viral DNA vectors adhere to stringent regulatory requirements for gene therapy medicinal products, including:

- Draft Guideline on the Quality, Non-Clinical and Clinical Aspects of Gene Therapy Medicinal Products (EMA/CAT/80183/2014, Committee for Advanced Therapies)

- Reflection Paper on Design Modifications of Gene Therapy Medicinal Products During Development (14 December 2011 EMA/CAT/GTWP/44236/2009 Committee for Advanced Therapies)

These DNA vectors were designed according to the FDA-2014-D-0663 guidelines to avoid impacting cellular proliferative activity, cell morphology, and reproductive health, as well as to prevent the transfer of antibiotic resistance genes into the environment.

Production and quality control—particularly with respect to safety parameters (endotoxin levels, sterility, stability, purity, biological activity, etc.)—are carried out in compliance with:

- U.S. Pharmacopeia (USP 42 and NF 37) Chapter 1047 (Gene Therapy Products)
- FDA-2008-D-0205 “Chemistry, Manufacturing, and Control (CMC) Information for Human Gene Therapy Investigational New Drug Applications (INDs) Guidance for Industry”
- FDA-2015-D-3399 “Recommendations for Microbial Vectors Used for Gene Therapy.”

Key Advantages of VTvaf17-Series Non-Viral DNA Vectors

1. Highest Level of Safety
    - Absence of viral regulatory elements: The DNA vectors do not contain nucleotide sequences derived from viral genomes.
    - No genomic integration: Unlike most viral vectors, non-viral DNA vectors remain in the cell as autonomous molecules rather than integrating into the chromosomes, mitigating potential negative outcomes such as oncogene activation or disruption of normal gene function.
    - No risk of spontaneous oncogenic transformation: This is linked to the absence of genomic integration and other oncogenic elements.
    - No antibiotic resistance genes: Consequently, there is no risk of creating antibiotic-resistant strains. For selection, VTvaf17 vectors include an RNA-out element (a regulatory component of the Tn10 transposon), enabling positive selection without antibiotics when used with a specialized Escherichia coli strain.
    - Targeted action: Specific promoters ensure gene expression only in the intended cell types, minimizing immune reactions and disruptions to normal cellular processes.
    - Encapsulation in a low-immunogenicity carrier: This helps avoid substantial immune responses against the vector DNA.
2. Maximum Efficacy
    - Incorporation of high-efficiency promoters into the vectors, enhancing gene expression in target cells.
    - Minimal vector size, enabling effective transfection of target tissue cells with no significant toxicity.

- Encapsulation within a transport system that shields the nucleic acids from extracellular nucleases, increasing stability in biological fluids; this optimally delivers the genetic material to the target cells.

IV. Gene Selection

The developer of this medicinal product, in collaboration with external experts and aided by specialized computational intelligence systems and algorithms, has compiled an initial (baseline) list of native human candidate genes capable of fulfilling the designated therapeutic functions.

To address the integrated task of Evaluating and Ranking Candidate Genes, a multi-round Analytic Hierarchy Process with Weighted Criteria (AHP) was employed. This method involves analyzing each therapeutic objective, ranking the genetic tools (candidate proteins) pertinent to these objectives independently, and then creating an aggregated (composite) ranking of candidate proteins.

For bioinformatic analysis, a scoring system was developed to rate “Benefit” (ranging from +100 to 0) and “Harm” (ranging from 0 to –100). This system is used both for prioritizing the therapeutic tasks themselves and for generating a final “weighted” score for each candidate gene. The Benefit score reflects how effectively the protein encoded by each gene influences the biological processes central to a given therapeutic objective; the Harm score accounts for the risks of oncogenic events, adverse effects, hyperexpression, or ectopic expression.

Below is the list of candidate genes/proteins subjected to bioinformatic assessment:

Gene / Protein	Family	Description
BMP-2	Bone Morphogenetic Proteins	A key inducer of osteoblast differentiation and bone tissue formation.
BMP-4	Bone Morphogenetic Proteins	Stimulates early development of bone and cartilage cells by activating osteogenic pathways.
BMP-6	Bone Morphogenetic Proteins	Enhances osteoblast differentiation and promotes mineralization of the bone matrix.
BMP-7	Bone Morphogenetic Proteins	Critical for bone and cartilage regeneration through activating osteogenic cell differentiation.
BMP-9	Bone Morphogenetic Proteins	Exhibits strong osteogenic activity, supporting bone tissue formation and remodeling.
TGF-β1	Transforming Growth Factor β	Regulates osteoblast proliferation and extracellular matrix synthesis in bone formation.
TGF-β2	Transforming Growth Factor β	Facilitates the recruitment of osteoprogenitor cells and formation of new bone tissue.
TGF-β3	Transforming Growth Factor β	Modulates osteoblast differentiation and synthesis of extracellular matrix components.
PDGF	Platelet-Derived Growth Factor	Attracts mesenchymal stem cells, stimulates angiogenesis, and contributes to bone healing.

IGF-1	Insulin-like Growth Factors	Accelerates osteoblast growth and differentiation, increases matrix synthesis and mineralization.
IGF-2	Insulin-like Growth Factors	Promotes bone growth and remodeling by stimulating osteoblast activity.
VEGF	Vascular Endothelial Growth Factor	Facilitates vessel formation, essential for nutrient and mineral delivery to bone tissue.
GDF-5	Growth/Differentiation Factors	Involved in joint and bone formation, supporting chondrogenesis and osteogenesis.
GDF-6	Growth/Differentiation Factors	Regulates development of skeletal and cartilaginous structures and proper "patterning" of the skeleton.
GDF-7	Growth/Differentiation Factors	Influences tendon, cartilage, and bone formation, supporting tissue repair.
COL1A1	Collagens	A principal structural protein of the bone matrix, providing strength and support.
COL1A2	Collagens	Together with COL1A1, forms type I collagen fibrils, ensuring the integrity of bone tissue.
COL3A1	Collagens	Participates in the early healing phase by creating a fibrous framework for subsequent bone regeneration.
COL5A1	Collagens	Regulates collagen fibril assembly and affects the organization of the bone matrix.
COL5A2	Collagens	Works alongside COL5A1 to control fibril diameter and structural integrity of the bone matrix.
COL10A1	Collagens	Associated with hypertrophic chondrocytes and involved in endochondral ossification.
BGLAP	Non-collagenous proteins	Known as osteocalcin; binds calcium and contributes to the mineralization of bone tissue.
SPP1	Non-collagenous proteins	Osteopontin; regulates cell-matrix interactions and supports bone remodeling.
SPARC	Non-collagenous proteins	Osteonectin; binds collagen and hydroxyapatite, playing a role in bone growth and mineralization.
IBSP	Non-collagenous proteins	Bone sialoprotein; responsible for cell-matrix adhesion and initiation of mineralization.
DCN	SLRPs (Small Leucine-Rich Proteoglycans)	Decorin; modulates collagen fibril formation and influences bone strength.
BGN	SLRPs	Biglycan; regulates assembly of the extracellular matrix and osteoblast activity.
FMOD	SLRPs	Fibromodulin; involved in the organization of collagen fibers and stabilization of the bone matrix.
FN1	Other Glycoproteins	Fibronectin; promotes cell adhesion and migration during the early stages of bone matrix formation.
LAM	Other Glycoproteins	Laminin; plays a key role in cell adhesion and basement membrane formation, essential in the early phases of osteogenesis.

Optimal Promoters

A separate bioinformatic analysis was performed to select optimal promoters by evaluating the efficacy/safety index of therapies using DNA vectors that encode target genes.

Key Considerations in Promoter Selection

- Target vs. non-target cells/tissues
- Specific conditions within the designated expression site

The selection of promoters was based on the ratio of effective expression level to the risks of ectopic expression and undesirable effects, as well as the duration of expression.

The following promoter options were considered:

1. Universal Promoter EF1α
   EF1α (Elongation Factor-1 alpha) is involved in transporting aminoacyl-tRNAs to ribosomes during translation and is expressed in virtually all eukaryotic cells. Owing to its strong and stable activity, EF1α is widely utilized in both transgenic and gene therapy constructs.

Advantages of EF1α:

- Constitutive Expression: Offers a stable, high-level transcription rate across a wide range of cells, including mesenchymal stem cells (MSCs).
- Applicability to Various Cell Cycle Stages: Functions in proliferating and non-proliferating cells, critical in tissues where some cells are dividing and others are quiescent. Given its broad-spectrum profile, EF1α can serve as a "universal" promoter across multiple cell populations in the bone system, from early-stage mesenchymal stem cells to more mature osteoblasts.

2. Promoters Specific to Mesenchymal Stem Cells (MSCs): MSCs can proliferate and differentiate into osteoblasts, chondroblasts, adipocytes, and other cell types. To deliver genes effectively into MSCs, researchers frequently employ non-viral, constitutive promoters that do not rely on viral enhancers.

   2.1 EF1α Promoter (revisited in the context of MSCs)

   - Role in MSCs: Provides stable expression immediately post-transfection/transduction, including during proliferation.
   - Advantage: Ensures rapid onset of the therapeutic effect owing to high baseline activity. *(EF1α is discussed above as a universal promoter, but here its specific relevance to MSCs is emphasized.)*

   2.2 PGK (Phosphoglycerate Kinase) Promoter

   - Specificity: PGK is a glycolytic enzyme expressed in many eukaryotic cell types, including MSCs.

- Advantage: Ensures robust, stable expression without being of viral origin.
- Comment: Commonly used in tissue engineering studies as one of the "universal" non-viral promoters, supporting reliable expression during long-term cell culture.

3. Promoters Specific to Osteoblasts: For selective expression of therapeutic genes in osteoblasts, promoters associated with bone tissue or osteogenic differentiation are employed.

3.1 Osteocalcin (BGLAP) Promoter

- Selectivity: Osteocalcin (BGLAP) is primarily synthesized by mature osteoblasts and is involved in bone mineralization.
- Advantage: Highly specific to post-proliferative osteoblasts, minimizing off-target expression.
- Significance: Elevates therapeutic gene expression precisely at the stage when cells transition to active mineralization of the bone matrix.

3.2 RUNX2 Promoter

- Selectivity: RUNX2 (Core-Binding Factor Alpha 1) is the principal transcription factor in osteogenic differentiation.
- Advantage: Drives expression in cells transitioning "from MSCs to osteoblasts," enabling gene activity even during early stages of bone cell formation.
- Significance: Amplifies osteogenic processes at the "onset" of differentiation, vital for expediting regenerative outcomes.

To maximize the efficacy of Genoterosil at the fracture site, it is critical to achieve transfection across the wide range of cells present in the fracture zone, including mesenchymal stem cells and osteoblasts in various developmental stages. The EF1α promoter provides a high and stable level of therapeutic gene expression in nearly all cell types—both actively proliferating and less active. This broad coverage significantly enhances the overall effectiveness of gene therapy and accelerates osteogenesis.

The minimization of ectopic expression will be addressed through the chosen method of drug administration, which aims to confine gene expression to the fracture site.

Therefore, EF1α has been chosen as the promoter in the gene therapy constructs designed to transfect cells at the fracture site.

Summary of Bioinformatic Analysis of Target Genes/Proteins

The following genes/proteins were included in the final (key) gene/protein list based on the results of processing using a three-round analytical hierarchy methodology:

Gene/Protein	Rationale for Inclusion	Secreted
BMP-2	One of the principal bone morphogenetic proteins (BMPs), actively stimulating the differentiation of mesenchymal stem cells into osteoblasts and contributing to the formation of a robust bone callus. BMP-2 underpins crucial stages of osteogenesis, from the initiation of organic matrix synthesis to its mineralization, significantly expediting the recovery process of bone tissue.	Yes
BMP-7	Demonstrates proven efficacy in bone tissue regeneration and is widely used in clinical settings to treat complex fractures and bone defects. BMP-7 enhances the proliferation and osteogenic differentiation of cells, promoting the development of a solid callus and boosting the mechanical strength of newly formed bone structure.	Yes
COL1A1	Encodes the primary chain of type I collagen, which constitutes up to 90% of the organic matrix in bone tissue. COL1A1 provides strength, elasticity, and proper organization of the bone matrix, laying the groundwork for effective mineralization and rapid formation of new bone tissue. Its adequate expression is one of the most critical factors for successful fracture healing.	Yes
COL1A2	An indispensable component of type I collagen structure, forming a triple helix together with COL1A1 to ensure the mechanical stability and durability of bone tissue. Without sufficient COL1A2, the forming collagen is weakened, adversely affecting regeneration and potentially resulting in an incomplete bone matrix or fibrous callus.	Yes

Therapeutic efficacy is attained through a **multiplicative impact**, owing to the inclusion of genes in the drug formulation that regulate the therapeutic objectives and associated biological processes defined in the Integral Task.

From a technical standpoint, this concept requires creating therapeutic non-viral DNA vectors based on the VTvaf17 platform for each protein/gene included in the final (key) list. The table below provides the list of these therapeutic DNA vectors:

DNA Vector	Therapeutic Gene	Promoter	Size, bp
VTvaf17-BMP-2	BMP-2	EF1α	4,360
VTvaf17-BMP-7	BMP-7	EF1α	4,463
VTvaf17-COL1A1	COL1A1	EF1α	7,563
VTvaf17-COL1A2	COL1A2	EF1α	7,269

Final Dosage Form (FDF) Selection

When determining the FDF, the following criteria were applied (in descending order of importance):

1. Therapeutic Efficacy; Minimization of Adverse Events

2. Maximal Preservation of Drug Functionality
3. Convenience of Administration, Transportation, and Storage
4. Acceptable Cost Parameters

Potential Dosage Forms

1. Lyophilized Complex of DNA Vectors with Calcium-Phosphate Precipitate

- Advantages
  - High Biocompatibility: Calcium phosphate closely resembles the mineral component of bone tissue (hydroxyapatite), reducing the risk of toxicity and allergic reactions.
  - Osteoconductive Properties: In addition to functioning as a delivery system, calcium phosphate contributes "building" ions (Ca^{2+} and PO_4^{3-}) that facilitate more effective mineralization of both primary (woven) bone and more organized (lamellar) bone. Intensive hydroxyapatite crystal formation within the collagen scaffold increases the mechanical strength of newly forming bone tissue.
  - Safety: Minimal cytotoxic effects, which is especially important when injecting the product directly into the defect site.
  - Ease of Storage and Transportation: Lyophilization ensures long-term stability, simplifying logistics and adherence to storage requirements.
- Disadvantages
  - Transfection efficiency may be slightly lower than certain polymer or liposomal systems, although optimization of particle size and surface properties can mitigate this issue.
  - Precise technological parameters must be maintained to ensure uniform adhesion between calcium-phosphate particles and DNA vectors.

2. Lyophilized Complex of DNA Vectors Encapsulated in Liposomes

- Advantages
  - High Transfection Efficiency: Liposomal systems generally deliver DNA effectively into cells through liposome–cell membrane fusion.
  - Broad Potential for Modification: Liposomes can be functionalized with various surface ligands (e.g., antibodies or peptides) to target specific cell types.
- Disadvantages
  - Toxicity: Although liposomes are typically considered biocompatible, some formulations (particularly cationic lipids) may elicit inflammatory responses or exhibit toxicity at high concentrations.

- Higher Production Costs: The liposome preparation process (extrusion, homogenization, microfluidic mixing, etc.) requires multi-stage quality control and specialized equipment, raising the final product's cost.
- Lack of Osteoinductivity: Liposomes do not inherently stimulate bone regeneration. Unlike calcium phosphate, which also supplies "building" ions (Ca^{2+} and PO_4^{3-}), their sole function is to facilitate cellular transfection.

3. Lyophilized Complex of DNA Vectors Encapsulated in Liposomes and PEG

- Advantages
  - High Transfection Efficiency: Liposomal and PEG-stabilized systems often permit effective DNA uptake by cells.
  - Extended Options for Modification: Liposomes can be further functionalized (e.g., with surface ligands for cell-type targeting).
- Disadvantages
  - Additional Toxic Effects: Dual encapsulation (liposome + PEG) may trigger inflammatory responses or compromise hemodynamics, particularly with local administration.
  - Higher Production Costs: Such multi-step technology demands complex equipment and quality controls, raising overall manufacturing costs.
  - No Osteoinductivity: Liposomes + PEG alone do not encourage bone regeneration; unlike calcium phosphate—which acts as a source of "building" ions (Ca^{2+} and PO_4^{3-})—these combinations only support cellular transfection.

4. Lyophilized Complex of DNA Vectors with the Transfection Reagent PEIpro (Polyethylenimine)

- Advantages
  - Good Transfection Activity: Polyethylenimine (PEI) can form stable complexes with DNA, enhancing gene transfer into cells in many contexts.
  - Well-Studied: PEI is widely used in clinical research; multiple modifications and protocols exist.
- Disadvantages
  - Toxicity: PEI can induce adverse effects, restricting its clinical utility, particularly at high volumes or during repeated administration.
  - No Osteoinductivity: Polyethylenimine itself does not promote bone regeneration. In contrast to calcium phosphate, which also provides "building" ions (Ca^{2+} and PO_4^{3-}), it only facilitates transfection.

Rationale for Selecting Calcium-Phosphate–Based Lyophilized Complex

Safety, biocompatibility, and functionality emerged as decisive criteria in choosing a delivery platform for Genoterosil. Calcium-phosphate precipitate is naturally compatible with bone tissue and possesses osteoconductive properties that enhance bone regeneration. Lyophilization affords convenient storage, transport and administration of the drug. Prior to administration, the powder must be diluted in the required solution in accordance with the protocol.

Consequently, the selected FDF is a DNA plus calcium-phosphate precipitate in lyophilized form, as it most effectively meets the requirements for safety, biocompatibility, and functionality in fracture treatment and bone tissue repair.

V. Delivery Methods

A range of administration strategies was evaluated for administering Genoterosil:

1. Single Intramuscular Injection Near the Fracture Site
    - Advantages:
        - Simplified procedure: one injection into the primary fracture area.
        - Reduced tissue trauma, as there is only one injection point.
    - Disadvantages:
        - In complex or extensive fractures, there is a risk of uneven distribution of the drug, potentially leaving peripheral regions of the defect underdosed.
        - Even with accurate placement, parts of the fracture site may fail to receive a sufficient concentration of osteogenic factors.
2. Multiple (4–6) Intramuscular Injections Near the Fracture Line. Administer the product as close as possible to the fracture line, selecting injection points based on fracture characteristics, accessibility, and avoidance of critical neurovascular bundles.
    - Advantages:
        - Comprehensive coverage of the fracture zone: multiple injection points ensure all critical areas receive the product.
        - Injections occur during a single procedure, retaining overall expression duration but significantly improving uniform distribution.
    - Disadvantages:
        - Greater volume of manipulation, as multiple injections may increase patient discomfort.
        - Requires meticulous planning of injection points to avoid overlap and excessive trauma to adjacent soft tissues, and to ensure vascular and neural safety.

Selecting the Optimal Delivery Approach

Two key objectives were considered when selecting the most appropriate method of drug delivery:

1. Guaranteed coverage of the entire fracture site, particularly in complex fractures where a single injection might fail to deliver an even concentration of the drug.
2. Minimization of systemic side effects via localized activity of gene therapy agents, maintaining the necessary amount of product throughout the different areas of the fracture.

Based on these considerations, multiple (4–6) intramuscular injections close to the fracture line were chosen. This approach ensures uniform dispersion of Genoterosil among the target cells in the fracture area and, by extending coverage, improves overall osteogenic efficacy while diminishing the risk of areas with insufficient gene expression to trigger regeneration.

Injection Control Methods

To increase accuracy in delivering the product to the fracture zone, image-based navigation is recommended:

1. X-Ray Guidance (Fluoroscopy)
   - Advantages:
     - Good visualization of bony structures in real time (using a mobile C-arm or a stationary X-ray machine).
     - Widespread availability and relative simplicity.
     - Facilitates precise needle navigation.
   - Disadvantages:
     - Radiation exposure for personnel and patients, albeit lower with modern equipment.
     - Limited detail regarding soft tissues and drug distribution.
2. Ultrasound (US) Navigation
   - Advantages:
     - No ionizing radiation (safer for staff and patients).
     - Good soft tissue and vascular detail, especially with Doppler.
     - Relatively low cost, portable equipment.
   - Disadvantages:
     - Bone obstructs ultrasound visualization, obscuring the fracture and deeper structures.
     - Operator skill is crucial, particularly for moving-needle guidance (requiring strong hand-eye coordination).
3. CT Guidance

- Advantages:
  - High-resolution, potentially 3D visualization of both bone and soft tissues.
  - Precise needle positioning, especially in complex fracture geometries.
  - Potential to track drug distribution with contrast agents or markers.
- Disadvantages:
  - Relatively high radiation dose (though low-dose protocols exist).
  - Expensive equipment, specialized personnel required.
  - Real-time needle monitoring is typically not feasible.

4. MRI Guidance
   - Advantages:
     - Excellent visualization of soft tissues, bone marrow changes (edema, inflammation), and optional contrast-labeled distribution.
     - No ionizing radiation.
   - Disadvantages:
     - High cost and technical complexity.
     - Longer procedure duration, requiring MRI-compatible instruments.
     - Limited availability; real-time needle monitoring is also not typically feasible.

Recommended Imaging Method

Real-time X-ray (fluoroscopic) guidance is advised, as it allows continuous monitoring of the fracture site and adjustment of injection depth/angle. Where possible, ultrasound can be used instead (particularly for superficial limb fractures) to reduce radiation exposure. However, one must be aware of the inherent limitations of ultrasound in terms of its ability to penetrate bony structures.

Additional Methods to Increase Transfection Efficiency

1. Ultrasonic Cavitation (Sonoporation)
   - Principle: Brief localized ultrasound exposure, combined with contrast microbubbles, generates “acoustic cavitation” in tissues. This increases cell membrane permeability, facilitating the entry of DNA vectors into the cytoplasm.
   - Considerations: Noninvasive, relatively targeted, but requires precise frequency/intensity/duration calibration to prevent tissue damage; also needs specialized ultrasound equipment.
2. Electroporation

- Principle: Short bursts of high- or low-voltage electrical pulses create transient pores in cell membranes, allowing DNA or other macromolecules to diffuse into cells.
- Considerations: Although highly effective, electroporation is constrained by the complexity of the equipment and patient discomfort. Suboptimal parameters risk tissue injury.

3. Moderate Mechanical Stimulation
   - Principle: Low-frequency vibration or mild static compression can stimulate osteogenesis through mechanosensors in osteocytes, osteoblasts, and stem cells. Slightly improved transfection efficiency might result from enhanced blood flow and marginally increased membrane permeability.
   - Considerations: Minimally invasive, physiologically compatible, but the direct effect on transfection rates is modest, requiring careful protocols for amplitude and frequency.
4. Controlled Local Hyperthermia
   - Principle: Elevating tissue temperature by 1–2 °C above normal can boost metabolic activity and endocytosis, potentially aiding gene transfer into cells. Often used in conjunction with heat shock proteins (HSP) for added cellular resilience and transcriptional modulation.
   - Considerations: Must monitor temperature and exposure duration to prevent tissue necrosis; specialized and costly setup may limit clinical adoption.

Taking into account noninvasiveness, clinical availability, and an established evidence base, ultrasonic cavitation (sonoporation) combined with low-intensity pulsed ultrasound (LIPUS) appears most promising. This protocol:

- Targets the ultrasound beam to a specific region, enhancing DNA vector uptake by cells.
- Minimizes tissue damage if parameters are carefully controlled.
- Utilizes LIPUS, already used clinically to hasten fracture healing.

Electroporation may deliver higher transfection rates, but it involves greater tissue trauma and more complex equipment, making it less practical in routine orthopedic settings. Moderate mechanical stimulation is beneficial as an adjunct but only mildly improves transfection. Controlled local hyperthermia may be viable, but tight temperature regulation and specialized infrastructure hinder widespread clinical use.

VI. Treatment Protocols

The therapeutic regimen can be adapted by adjusting the schedule and dosage of the drug, depending on both efficacy and potential adverse events:

- Duration: The drug exhibits a sufficient (~10-day) period of action (gene expression) in target cells.
- Frequency: Multiple administrations are feasible due to the lack of immunogenicity.
- Dosage: Personalized therapeutic protocols can be designed according to actual efficacy and side effects.

Depending on fracture characteristics, therapeutic objectives, and the drug pharmacokinetics, different administration regimens may be implemented. The principal options are:

1. Single Injection

- Advantages
  - Minimally invasive and convenient for the patient (only one procedure).
  - No need for repeated procedures or ongoing supervision.
- Limitations
  - Risk of insufficient gene expression duration: activity may wane, making healing less effective in severe fractures.
  - Unsuitable for extensive or chronic bone defects that require a prolonged osteogenic stimulus.

2. Two Injections (1–2 Week Interval)

- Typical Schedule
  - First injection on Day 5–9 post-surgery.
  - Second injection 7–14 days later, depending on clinical status.
- Advantages
  - Additional prolongation of effect: the second dose “reinforces” gene expression once growth-factor synthesis begins to decline.
  - Reduced likelihood of a “drop-off” in the concentration of osteogenic factors between healing periods.
- Limitations
  - May still be inadequate for slow-healing fractures or large bone defects.
  - In the presence of intense inflammation or complications, a third dose may be necessary.

3. Weekly Triple Injection (6-Day Interval)

- Advantages
  - Stable Expression: Preclinical animal models have shown that gene expression remains at a high level for about 7 days, then gradually tapers off. A second and third injection “refresh” expression, restoring secreted target proteins to a therapeutically significant level.

- Support During Critical Stages: The first three weeks post-fracture involve forming a soft (cartilaginous) callus followed by a hard (bony) callus. Triple administration substantially accelerates and amplifies this process.
- Balanced Treatment Burden: Only three procedures, minimizing patient discomfort while maintaining an extended therapeutic window.

4. Extended Regimen (Four or More Injections). Four to five doses administered at 6-day intervals. Potentially useful for severe or chronic fractures and significant bone defects with a high risk of delayed union or pseudarthrosis.

- Advantages
  - Prolonged, repeated stimulation supports all stages of bone healing (from inflammation to remodeling).
- Limitations
  - Significantly increases patient burden (multiple procedures) and overall treatment costs.
  - Likely unnecessary for most routine clinical scenarios where three injections suffice.

Final Choice: Weekly Triple Injection at 6-Day Intervals

Justification:

- Preclinical evidence confirms a 6-day interval to be optimal for reinforcing diminishing gene expression.
- Three separate doses align with the phases of active bone remodeling (from soft to hard callus formation).
- Amplified expression of target proteins during the active treatment period successfully initiates core osteogenic processes; subsequent formation of lamellar bone proceeds under standard follow-up care and rehabilitation.

Thus, a triple weekly administration regimen at 6-day intervals optimally balances therapeutic efficacy (continuous support of gene expression) with a moderate burden on the patient, as confirmed by preclinical research.

Recommended Dosage

Based on studies of specific pharmacological activity conducted in rabbits and Phase I clinical trials, a single-dose regimen of Genoterosil containing 1 mg of active substance in 2 mL of solvent (i.e., 0.014 mg/kg body weight for a 70 kg adult) is deemed both effective and safe.

VII. Economic Feasibility

Expert opinion suggests that upon successful completion of clinical trials and subsequent market entry, the economic prospects for the holder of the marketing authorization for this therapeutic product are highly favorable. Key considerations in the economic evaluation include:

- Absence of Direct Competitors: As of the date of this material, no directly competing medicinal product exists in this therapeutic space.
- Significant Patient Population in Patented Territories (USA, China, EU, and Russia):
  - These four regions account for over two billion people in total.
  - The average fracture incidence is roughly 1% annually, implying a potential patient population of around 20 million cases per year (excluding dental indications).
- Impressive Potential to Expand the Indications for the Drug: The product could be applied to most types of bone fractures, with the possibility of extending indications into dental medicine.
- Well-Established Manufacturing Technologies: The project uses proven technological approaches, facilitating production on a range of standard (non-specialized) biotechnology platforms. This ensures both competitive pricing and robust margins for the marketing authorization holder.
- Convenient Logistics and Storage: The developed drug's stable formulation allows for widespread use in diverse geographic settings.

By employing:

- Modern biomimetic molecular-genetic tools (unique non-viral DNA vectors),
- Comprehensive bioinformatics algorithms for therapeutic design (evaluation and ranking of candidate genes/proteins using a multi-round analytical hierarchy method, assessment of delivery methods, dosage form composition, etc.),
- Combinatorial strategies (optimal selection of genes/proteins; specificity tools for delivering the therapeutic agent to target cells; optimization of the dosage form, administration methods, and potential use of additional imaging techniques),

—and taking into account the aforementioned market factors, participants in this project have enhanced prospects for successfully commercializing this novel therapeutic product.

Completed Milestones

1. Bioinformatic Analysis: Identification of the Baseline List of Candidate Genes/Proteins Through extensive bioinformatic studies involving external molecular biology experts and advanced algorithms, an initial (baseline) list of candidate genes/proteins was compiled. These genes/proteins regulate osteogenic signaling pathways and enhance structural protein expression.

2. Bioinformatic Analysis: Ranking and Creation of the Key Candidate DNA Vector List
   The baseline list of candidate genes/proteins was ranked using a multi-round Analytic Hierarchy Process with weighted criteria (AHP). Each therapeutic task was analyzed separately, and genetic tools (candidate proteins) were ranked accordingly, producing a consolidated (integrated) list of top-tier proteins.
   The final composition includes BMP-2, BMP-7, COL1A1, COL1A2.

3. Creation of DNA Vectors
   - A platform DNA vector of the VTvaf17 series was developed.
   - Therapeutic DNA vectors based on the VTvaf17 platform were produced: VTvaf17-BMP-2, VTvaf17-BMP-7, VTvaf17-COL1A1, VTvaf17-COL1A2.

4. Depositing Producer Strains
   Original production strains for manufacturing these therapeutic DNA vectors have been deposited in international repositories (NCIMB, VKPM):
   - *Escherichia coli* SCS110-AF/VTvaf17-COL1A1,
   - *Escherichia coli* SCS110-AF/VTvaf17-COL1A2,
   - *Escherichia coli* SCS110-AF/VTvaf17-BMP2,
   - *Escherichia coli* SCS110-AF/VTvaf17-BMP7.

5. Registration of Intellectual Property
   All necessary patent procedures have been completed to secure IP rights for the VTvaf17 platform vectors, the therapeutic DNA vectors, and their associated producer strains in the USA, EU, China, and Russia. Patent references include:
   - USA: US 11,352,639 B2
   - China: ZL201980073044.8
   - EU: EP3847259
   - Russia: RU0002707537

6. Manufacturing and Research
   - Preliminary in vitro studies on platform DNA vectors were carried out, alongside the development of manufacturing processes and laboratory/pilot-scale production protocols.
   - The efficacy of an active gene-therapeutic substance containing BMP-2, BMP-7, COL1A1, and COL1A2 was evaluated in vitro on human osteoblast cultures, confirming its effectiveness.
   - A study evaluating the induction of ectopic bone tissue by the gene therapy DNA vector encoding BMP-2, BMP-7, COL1A1, and COL1A2 revealed no formation of

ectopic bone in laboratory animals, a significant advantage over existing recombinant-factor-based osteogenesis-stimulating therapies.

7. Finished Dosage Form (FDF) Development
    - The calcium-phosphate precipitate transport system was selected. In vitro and in vivo experiments assessed the efficiency of transfection/expression of target genes.
    - A lyophilized DNA–calcium phosphate complex was formulated as the preferred FDF.
    - Pilot-scale manufacturing protocols for both DNA vectors and the FDF were established.
8. Selection of Administration Method and Treatment Regimen
    - A comparative review of administration routes identified multiple (4–6) intramuscular injections near the fracture line as the optimal method.
    - Recommended imaging techniques—fluoroscopic (X-ray) guidance and ultrasound (where applicable)—were selected.
    - A weekly triple-dose regimen with 6-day intervals was established as the standard treatment protocol.
9. Dosage Determination
    - Based on specific pharmacological activity studies in rabbits and Phase I clinical trials, a single-dose strength of 1 mg of active ingredient in 2 mL of solvent (0.014 mg/kg for a 70 kg adult) was determined to be both effective and safe.
10. Comprehensive Preclinical Studies Confirming Efficacy and Safety

- *Allergenicity Assessment*: Demonstrated no allergenic effects.
- *Immunotoxicity Study*: Demonstrated no immunotoxic effects.
- *Mutagenicity Study*: Demonstrated no mutagenic effects.
- *Acute Toxicity Study*: Demonstrated low acute toxicity.
- *Reproductive Toxicity Study*: Demonstrated no gonadotoxic effects.
- *Pharmacokinetics (Biodistribution)*: Showed optimal distribution characteristics.
- *Chronic Toxicity Studies* in rats, rabbits, and primates: Demonstrated low toxicity; no fatalities or evidence of intoxication.
- *Efficacy Study in Difficult-to-Heal Fracture Models*: Demonstrated faster and higher-quality fracture healing than in control groups, evident from earlier cartilage-to-bone transition and more mature bone tissue by Day 30.

11. Optimization of Manufacturing Technology under GMP Conditions
    The production process has been validated at a GMP-certified facility, ensuring technology transfer capabilities to another specialized pharmaceutical plant if necessary.

12. Completion of Phase I Clinical Trials in Healthy Volunteers

- A favorable safety profile was confirmed.
- Approval was granted to move forward with combined Phase II–III trials.

Future Steps

- Additional Clinical Studies to evaluate efficacy and determine dosage optimization.
- Study Management: Data collection, database creation, statistical analyses, and final reporting.
- Technology Transfer of DNA vector and FDF manufacturing to an industrial-scale pharmaceutical enterprise.
- Pharmaceutical Quality Examination of the final product.
- Registration and Market Launch of the medicinal product.
- Refinement and Formalization of technological aspects of manufacturing methods on an industrial scale (validation of production, validation of quality control methods, report on the stability of the drug during storage, reporting on the system of strain-producer banks, etc.).
- Expanding the indication for use and increased patient coverage through broader clinical applications.