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CAN NEW GENE THERAPIES RESULT IN UNINTENDED GEMLINE MODIFICATION?

UNVEILING THE FRONTIRES: CURRENT INSIGHTS AND LIMITS OF TESTING

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Can new gene therapies result in unintended germline modification?

Unveiling the Frontiers: Current Insights and Limits of Testing

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COGEM Report CGM 2025-03

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The authors gratefully acknowledge the members of the advisory committee for the valuable discussions and patience.

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Disclaimer

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Dit rapport is samengesteld in opdracht van de COGEM en CCMO. De mening die in het rapport wordt weergegeven is die van de auteurs en weerspiegelt niet noodzakelijkerwijs de mening van de COGEM en CCMO.

Foreword

Gene therapies have the potential to cure or alleviate suffering due to heritable diseases, as well as some types of cancer and infectious conditions. Unmet medical needs may be met by newly developed products. The number of gene therapy products that has received market authorization is increasing, and new delivery methods are investigated. These therapies intend to treat the individual person, not their future children. In European and international legislation, heritable gene editing affecting future generations is not allowed. The question therefore surfaces whether therapies intended for somatic gene therapy could also lead to unintended germline modification. Some gene therapies are applied on cells outside of the body (*ex vivo*), but the potential for germline modification seems relevant especially for in vivo gene editing. Especially for new delivery methods (e.g. CRISPR therapies in lipid nanoparticles) developed for in vivo gene editing, there is uncertainty whether these could affect the germline.

COGEM and CCMO have commissioned a research project to assess the potential for inadvertent germline transformation associated with emerging gene therapy technologies. The project was carried out by Perseus and Sciensano who thoroughly evaluated the literature, investigated ongoing research projects and interviewed experts with regulatory, research and clinical background. None of the studies reported confirmed germline modification, but many uncertainties remain. Suggested approaches to fill this evidence gap by studying the potential for germline transmission are also discussed.
The advisory committee meetings with Perseus and Sciensano were insightful and pleasant. The advisory committee is pleased with the resulting report which provides an in-depth overview of the current knowledge on unintended germline modification as well as suggestions to fill the evidence gaps, taking into account both potential benefits and risks.

The study results are presented in this report. The advisory committee greatly appreciated the pleasant and constructive interaction with the authors. The committee is pleased that the authors succeeded in generating an overview of relevant information and regulatory aspects, which will be valuable for COGEM, CCMO and others interested in somatic gene therapies and mitigation of their potential future risks.

Finally I want to thank the members of the advisory committee for their constructive collaboration and feedback.

Martina C. Cornel
Chair of the Advisory Committee

Summary

Gene therapy represents a significant advancement in the treatment of genetic and rare disorders, offering the potential for durable and curative therapeutic outcomes. While current therapies are designed to target somatic cells, the rapid evolution of delivery technologies - particularly non-viral platforms - has prompted renewed scrutiny regarding the possibility of unintended germline modification, which could result in heritable genetic changes. Importantly, germline modification is explicitly prohibited under European Union legislation and international conventions, reinforcing the need for rigorous safety evaluation and regulatory oversight.

This report, commissioned by the Netherlands Commission on Genetic Modification (COGEM) and Central Committee on Research Involving Human Subjects (CCMO), aims to assess the potential for inadvertent germline transformation associated with emerging gene therapy technologies. The study focuses on *in vivo* applications and excludes *ex vivo* cell therapies and intentional germline editing. The primary objectives are to:

- Evaluate the biodistribution of gene therapy delivery systems to gonadal tissues.
- Examine biological barriers in the male and female reproductive systems.
- Review current scientific literature and clinical data.
- Analyse governance frameworks and regulatory guidance.
- Propose a structured approach to risk assessment.

This investigation employed a comprehensive approach to assess the potential for unintended germline modification in gene therapy. A structured literature review was conducted across major biomedical databases - PubMed, Embase, and Web of Science - yielding a curated dataset of 440 unique studies, primarily comprising preclinical data. In parallel, expert consultations provided contextual insights and helped identify knowledge gaps. This dual strategy provided a robust evaluation of the current evidence base and informed the conclusions presented in this report.

- **No Documented Evidence of Germline Modification**
  Despite theoretical concerns, there is currently no empirical evidence of unintended germline modification resulting from somatic gene therapy in either animal models or human clinical trials. While some delivery systems have been shown to reach reproductive tissues in preclinical studies (in mice, rabbits and occasionally also in non-human primates), no study has reported confirmed cases of germline modification.

- **Delivery Systems and Biological Barriers**
  Viral vectors such as adeno-associated virus (AAV), lentivirus, and adenovirus are well-characterized and generally pose a low theoretical risk of germline integration, although episomal persistence in gonadal tissues has occasionally been observed for AAV. Non-viral platforms - including lipid nanoparticles, extracellular vesicles, liposomes, and polymers - are increasingly used due to their transient expression

and reduced immunogenicity, yet their biodistribution profiles remain less well understood. Importantly, the anatomical and physiological features of the reproductive systems - particularly the blood-testis barrier (BTB), blood-epididymis barrier (BEB), and blood-follicle barrier (BFB) - serve as intrinsic safeguards, significantly limiting the exposure of germ cells, spermatozoa, and oocytes to therapeutic delivery systems.

- **Biodistribution Data are Central to Risk Assessment**
  As gene therapy technologies diversify, biodistribution assessment strategies must evolve to remain scientifically relevant. Viral vectors, due to their potential for genomic integration or episomal persistence, allow for longer-term tracking using nucleic acid-based methods. In contrast, non-viral delivery systems typically do not leave nucleic -acid based remnants and mostly induce transient expression, thereby requiring highly sensitive detection techniques within narrow timeframes to capture their biodistribution. Particularly, in gene-editing applications where permanent genomic changes may occur despite rapid degradation of editing components, additional methods should be considered to detect unintended germline modification, like deep sequencing of germline tissues or genetic analysis of offspring.

- **Focussing the Risk Assessment Based on Therapeutic Approach**
  Regulatory authorities advocate a tiered, risk-based framework for evaluating the potential for unintended germline modification. A key determinant in this assessment is the presence or absence of delivery system material in gonadal tissues; however, negative findings must be interpreted with caution due to limitations in analytical sensitivity and biological relevance. Given the diversity in mechanisms of action and integration potential of gene therapy platforms, a more refined classification system - based on delivery system biodistribution, cellular localization and nature of the payload/cargo - can improve the precision and consistency of risk evaluations. While existing regulatory frameworks provide a solid foundation, they require updates to adequately address emerging technologies, particularly non-viral delivery systems and gene-editing platforms.

- **Transparency and Data Sharing Are Essential**
  Publicly available data on the biodistribution of gene therapy delivery systems to gonadal tissues remain limited and fragmented, in particular for non-viral platforms that are still in early development. Many relevant findings are unpublished or proprietary, hindering comprehensive risk evaluation. To support informed regulatory decisions and maintain public trust, greater transparency in preclinical and clinical data is essential. Continued research and harmonized guidance are needed to enable robust, case-by-case assessments of germline safety.

- **Balancing Risk and Benefit Is Crucial**
  While hypothetical risks of unintended germline modification must be carefully considered, they should be weighed against the potential therapeutic benefits for

patients of the intended somatic modification. To date, no evidence supports the occurrence of germline modification following somatic gene therapy; however, this absence of evidence does not eliminate the possibility of rare events. A precautionary yet adaptable regulatory approach is therefore essential to ensure safety without unnecessarily delaying access to promising treatments. Strengthening methodological standards, improving data availability, and implementing proportionate risk mitigation measures - such as reproductive precautions including effective contraception requirements and/or considering oocyte cryopreservation or sperm donation prior to gene therapy - can support responsible innovation. Establishing standardized risk assessment frameworks can help researchers evaluate and communicate potential hazards more consistently, while regulators' confidence can be enhanced through reliance on validated and reproducible preclinical tests. A flowchart **(Figure 4**) presented in this report's conclusion offers a balanced overview for non-clinical assessment of investigational somatic gene therapy medicinal products, complementing this report's chapters and guiding risk-benefit evaluation. The use of platform technologies with predictable behaviour may further streamline safety evaluations, facilitate consistent regulatory oversight, and support mutual trust between researchers and regulators.

Samenvatting

Gentherapie vertegenwoordigt een significante vooruitgang in de behandeling van genetische en zeldzame aandoeningen, met potentieel voor duurzame en curatieve therapeutische uitkomsten. Hoewel de huidige toepassingen gericht zijn op somatische cellen, heeft de snelle ontwikkeling van toedieningstechnologieën - in het bijzonder niet-virale platforms - geleid tot hernieuwde aandacht voor het risico op onbedoelde kiembaanmodificatie, hetgeen zou kunnen resulteren in erfelijke genetische veranderingen. Het is van belang te benadrukken dat kiembaanmodificatie expliciet verboden is binnen de wetgeving van de Europese Unie en diverse internationale verdragen, hetgeen de noodzaak onderstreept van rigoureuze veiligheidsevaluaties en strikt regelgevend toezicht.

Dit rapport, opgesteld in opdracht van de Commissie Genetische Modificatie (COGEM) en de Centrale Commissie Mensgebonden onderzoek (CCMO), heeft tot doel het potentieel van onbedoelde kiembaantransformatie in verband met opkomende gentherapietechnologieën te beoordelen. De studie richt zich op *in vivo*-toepassingen en sluit *ex vivo*-celtherapieën en opzettelijke kiembaanmodificatie uit. De belangrijkste doelstellingen zijn:

- De biodistributie van het afleveringssysteem voor genetische materiaal naar gonadaal weefsel te evalueren.
- Biologische barrières in het mannelijke en vrouwelijke voortplantingssysteem te onderzoeken.
- De huidige wetenschappelijke literatuur en klinische gegevens te beoordelen.
- Bestuurskaders en regelgevende richtsnoeren te analyseren.
- Een gestructureerde aanpak voor risicobeoordeling voor te stellen.

Voor dit onderzoek werd een geïntegreerde benadering gehanteerd om het potentieel voor onbedoelde kiembaanmodificatie bij gentherapie te beoordelen. Een gestructureerd literatuuronderzoek in toonaangevende biomedische databases - PubMed, Embase en Web of Science - resulteerde in een zorgvuldig samengestelde dataset van 440 unieke studies, voornamelijk bestaande uit preklinische gegevens. Parallel hieraan verschaften consultaties met deskundigen aanvullende context en droegen bij aan het identificeren van kennislacunes. Deze gecombineerde aanpak bood een solide basis voor een grondige evaluatie van de beschikbare evidentie en vormde het fundament voor de conclusies in dit rapport.

- **Geen Gedocumenteerd Bewijs van Kiembaanmodificatie**
  Ondanks theoretische bezorgdheden is er momenteel geen empirisch bewijs van onbedoelde kiembaanmodificatie als gevolg van somatische gentherapie, noch in diermodellen (muizen, konijnen, en in enkele gevallen ook in niet-humane primaten), noch in klinische studies bij mensen. Hoewel in preklinische studies is aangetoond dat sommige afleveringssystemen reproductieve weefsels bereiken, heeft geen enkele studie bevestigde gevallen van kiembaanmodificatie gemeld.

- **Toedieningssystemen en Biologische Barrières**
  Virale vectoren zoals adeno-geassocieerd virussen (AAV), lentivirussen en adenovirussen zijn goed gekarakteriseerd en vormen over het algemeen een laag theoretisch risico op kiembaanintegratie, hoewel bij AAV af en toe episomale persistentie in geslachtsklierweefsels is waargenomen. Niet-virale platforms, waaronder lipide-nanodeeltjes, extracellulaire vesikels, liposomen en polymeren, worden steeds vaker gebruikt vanwege hun tijdelijke expressie en verminderde immunogeniciteit, maar hun biodistributieprofielen zijn minder goed bekend. Belangrijk is dat de anatomische en fysiologische kenmerken van de voortplantingssystemen - met name de bloed-testisbarrière (BTB), de bloed-epididymisbarrière (BEB) en de bloed-follikelbarrière (BFB) - als intrinsieke beschermingsmechanismen fungeren, waardoor de blootstelling van kiemcellen, spermatozoa en eicellen aan therapeutische afleveringssystemen aanzienlijk wordt beperkt.

- **Biodistributiegegevens Zijn Cruciaal voor Risicobeoordeling**
  Naarmate gentherapietechnologieën zich verder ontwikkelen, dienen ook de strategieën voor het beoordelen van biodistributie mee te evolueren om wetenschappelijk relevant te blijven. Virale vectoren maken, vanwege hun vermogen tot genomische integratie of episomale persistentie, langdurige monitoring mogelijk via nucleïnezuurgebaseerde methoden. Daarentegen veroorzaken niet-virale platforms doorgaans een voorbijgaande expressie, waardoor zeer gevoelige detectietechnieken binnen beperkte tijdsvensters vereist zijn. Dit geldt in het bijzonder voor toepassingen in genbewerking, waarbij permanente genomische veranderingen kunnen optreden ondanks de snelle afbraak van de bewerkingscomponenten. In zulke gevallen dienen aanvullende methoden te worden overwogen om onbedoelde kiembaanmodificaties te detecteren, zoals diepgaande sequencing van kiembaanweefsels of genetische analyse van nakomelingen.

Risicobeoordeling Toegespitst op Basis van de Therapeutische Aanpak
Regelgevende instanties pleiten voor een gelaagd, risico-gebaseerd kader voor de beoordeling van het potentieel voor onbedoelde kiembaanmodificatie. Een belangrijke bepalende factor bij deze beoordeling is de aanwezigheid of afwezigheid van afleveringssysteem materiaal in gonadaal weefsel; negatieve bevindingen moeten echter met voorzichtigheid worden geïnterpreteerd vanwege beperkingen in de analytische gevoeligheid en biologische relevantie. Gezien de diversiteit in werkingsmechanismen en integratiepotentieel van gentherapieplatforms, kan een verfijnder classificatiesysteem – gebaseerd op biodistributie van het afleveringssysteem, cellulaire lokalisatie en aard van de lading – de nauwkeurigheid en consistentie van risicobeoordelingen verbeteren. Hoewel bestaande regelgevingskaders een solide basis vormen, moeten ze worden bijgewerkt om adequaat in te spelen op opkomende technologieën, met name niet-virale toedieningssystemen en platforms voor genbewerking.

- **Transparantie en Gegevensuitwisseling Zijn Essentieel**
  Openbaar beschikbare gegevens over de biodistributie van afleveringssystemen voor gentherapie naar gonadaal weefsel blijven beperkt en gefragmenteerd, met name voor niet-virale platforms die zich nog in een vroeg ontwikkelingsstadium bevinden. Veel relevante bevindingen zijn niet gepubliceerd of zijn eigendom van bedrijven, wat een uitgebreide risicobeoordeling belemmert. Om weloverwogen regelgevings-beslissingen te ondersteunen en het vertrouwen van het publiek te behouden, is meer transparantie van preklinische en klinische gegevens essentieel. Voortgezet onderzoek en geharmoniseerde richtsnoeren zijn nodig om een robuuste, per geval beoordeling van de veiligheid van kiembaanweefsels mogelijk te maken.

- **Het Afwegen van Risico's en Voordelen Is Cruciaal**
  Hoewel hypothetische risico's van onbedoelde kiembaanmodificatie zorgvuldig moeten worden afgewogen, moeten deze worden afgewogen tegen de potentiële therapeutische voordelen voor patiënten van de beoogde somatische modificatie. Tot op heden zijn er geen aanwijzingen dat somatische gentherapie leidt tot kiembaanmodificatie, maar dit betekent niet dat zeldzame gevallen volledig kunnen worden uitgesloten. Een voorzorgsgerichte maar flexibele regelgevingsaanpak is essentieel om de veiligheid te waarborgen zonder de toegang tot veelbelovende behandelingen onnodig te vertragen. Versterking van methodologische normen, verbetering van de beschikbaarheid van gegevens en de invoering van evenredige risicobeperkende maatregelen - zoals reproductieve voorzorgsmaatregelen, waaronder het vereisen van effectieve anticonceptie en/of het overwegen van oöcytcryopreservatie of zaaddonatie voorafgaand aan gentherapie. Het opstellen van gestandaardiseerde risicobeoordelingskaders kan onderzoekers helpen om potentiële risico's consistenter te evalueren en te communiceren, terwijl het vertrouwen van toezichthouders kan worden vergroot door gebruik te maken van gevalideerde en reproduceerbare preklinische testen. Een stroomdiagram **(Figure 4**) in de conclusie van dit rapport biedt een evenwichtig overzicht voor de niet-klinische beoordeling van somatische gentherapie-geneesmiddelen in onderzoek, als aanvulling op de hoofdstukken van dit rapport en ter ondersteuning van de risico-batenafweging. Het inzetten van platformtechnologieën met voorspelbaar gedrag kan de veiligheidsevaluaties verder stroomlijnen, consistente regulatoire toetsing vergemakkelijken en het wederzijdse vertrouwen tussen onderzoekers en toezichthouders versterken.

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Abbreviations

3R	Replacement, Reduction, Refinement
AATD	Alpha-1 antitrypsin deficiency
AAV	Adeno-associated virus
AAV5	AAV serotype 5
ACE2	Angiotensin I-converting enzyme 2
ASCVD	Atherosclerotic cardiovascular disease
ATMP	Advanced therapy medicinal product
ATTR-CM	Transthyretin amyloidosis with cardiomyopathy
ATTR-PN	Hereditary transthyretin amyloidosis with polyneuropathy
AuNPs	Gold nanoparticles
BD	Biodistribution
BEB	Blood-epididymis barrier
BFB	Blood-follicle barrier
BGGO	Office for Genetically Modified Organisms (GMO Office) (The Netherlands) "*Bureau Genetisch Gemodificeerde Organismen (Bureau GGO)*"
BLI	Bioluminescence imaging
BTB	Blood-testis barrier
CCMO	Central Committee on Research Involving Human Subjects (The Netherlands) "*Centrale Commissie Mensgebonden Onderzoek*"
CGTP	Cell and gene therapy products
CNS	Central nervous system
COGEM	Commission on Genetic Modification (The Netherlands) "*Commissie Genetische Modificatie*"
CPPs	Cell-penetrating peptides
CRISPR	Clustered regularly interspaced short palindromic repeats
CRISPR-Cas	Clustered Regularly Interspaced Short Palindromic Repeats–CRISPR-associated proteins
CT	Computed tomography
DART	Development and reproductive toxicology studies
ddPCR	Droplet digital polymerase chain reaction
DNA	Deoxyribonucleic acid
EFD	Embryo-foetal development
EMA	European Medicines Agency
ERIS	European Research Information System
EU	European Union
EVs	Extracellular vesicles
FAST	Fusion-associated small transmembrane
FDA	Food and Drug Administration

FRT	Female reproductive tract
GAG	Glycosaminoglycans
GCT	Gene and cell therapies
GE	Genome editing
GFP	Green fluorescent protein
GLP	Good laboratory practice
GM	Genetically modified
GSD 1a	Glycogen storage disease 1a
HAE	Hereditary angioedema
hATTR	Hereditary transthyretin amyloidosis
HCT	Human cells and Tissues
HIV	Human immunodeficiency virus
HSV-1	Herpes simples virus type 1
IHC	Immunohistochemistry
ICH	International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use
ID	Injected dose
IL	Interleukin
IMP	Investigational medicinal product
ISH	*In situ* hybridization
IV	Intravenous
IVIS	*In vivo* imaging system
LCA10	Leber congenital amaurosis type 10
LNP	Lipid nanoparticles
LOD	Limit of detection
LSC	Liquid scintillation counting
LTFU	Long term follow-up
MAA	Marketing authorisation application
MEFL	Malformation or embryo-foetal lethality
MeSH	Medical Subject Headings
miRNA	MicroRNA
MNPs	Magnetic nanoparticles
MRI	Magnetic resonance imaging
mRNA	Messenger RNA
nAMD	Neovascular age-related macular degeneration
NAM	New approach methodologies
NAT	Nucleic acid amplification techniques
NHP	Non-humane primate
NIH	National institutes of health
OOC	Organ-on-a-chip

OECD	Organisation for economic co-operation and development
PBAE	Poly(beta-amino esters)
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PEI	Polyethyleneimine
Pep-NPs	Peptide-based nanoparticles
PET	Positron emission tomography
PK	Pharmacokinetic
PLGA	Poly(lactic-co-glycolic acid)
PLVs	Proteolipid vehicles
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
RIVM	Rijksinstituut voor volksgezondheid en milieu
ROA	Route of administration
RTK	Receptor tyrosine kinase
rtPCR	Real-time polymerase chain reaction
saRNA	Single stranded RNA
SB	Sleeping beauty
SC	Sertoli cells
SgRNA	Single guide RNA
siRNA	Small interfering RNA
SPECT	Single-photon emission computed tomography
SPECT/CT	Single-photon emission computed tomography and computed tomography
SSC	Spermatogonial stem cells
TEM	Transmission electron microscopy
TM	Testicular macrophage
TMPRSS2	Transmembrane serine protease 2
TNF	Tumor necrosis factor
TTR	Transthyretin
UK	United Kingdom
US	United States
VLP	Virus-like particles
WHO	World Health Organization

Glossary

Biodistribution	Biodistribution is the *in vivo* distribution, persistence, and clearance of a gene therapy product at the site of administration and in target and non-target tissues, including biofluids (e.g., blood, cerebrospinal fluid, vitreous fluid).
Cargo	In gene therapy, the complete set of therapeutic and supporting materials - such as DNA, RNA, or gene-editing components - encapsulated within a delivery system (e.g., lipid nanoparticles or viral vectors) for transport into target cells. The cargo includes the payload (*see below*) along with any additional sequences or modifications needed for stability, expression, or regulation.
CRISPR-Cas	CRISPR-Cas stands for Clustered Regularly Interspaced Short Palindromic Repeats–CRISPR-associated proteins, a genome editing technology that enables precise, targeted modifications to DNA within living organisms. In this report, CRISPR-Cas is highlighted as an example of a system that modifies the person's own genetic material, aiming to edit endogenous DNA rather than introduce or express an external gene function.
Episome	In gene therapy, an episome is a DNA molecule that exists and replicates independently of the host cell's chromosomal DNA. Episomes remain in the nucleus without integrating into the genome, thereby reducing the risk of insertional mutagenesis. Many viral vectors, such as adeno-associated virus (AAV), persist in target cells as episomes, enabling long-term but typically non-permanent expression of the therapeutic payload.
Gene editing	A set of techniques enabling precise, targeted modifications to an organism's DNA sequence, such as insertion, deletion, or replacement of genetic material, often using tools like CRISPR-Cas systems
in vivo & *ex vivo* gene therapy	*In vivo* gene therapy involves delivering genetic material directly into a person's body to target cells internally. *Ex vivo* gene therapy involves removing cells from a person, modifying them with the desired gene outside the body, and then returning them to the same or more persons.
Off-target effects	In gene therapy, unintended effects can occur at different levels. Off-target tissue effects refer to the delivery or activity of the therapeutic agent in non-target cell types or tissues, where the treatment was not intended to act. In contrast, off-target genomic effects involve unintended genetic modifications within the genome, such as edits at incorrect DNA sequences. The distinction is indicated in the report to clarify whether the issue lies in the location of the therapy's action or in the specificity of its genetic impact.
Payload	In gene therapy, a payload gene is the therapeutic gene introduced into cells to correct or replace a defective gene or to provide a new function. It is distinct from the delivery system, which is the vehicle - such as a viral vector or nanoparticle - used to introduce the payload gene into the target cells.
Replication-deficient viral vector	A viral vector in which the virus has been genetically modified so that it cannot reproduce itself in normal host cells. Replication-deficient viral vectors retain the ability to enter target cells and deliver their genetic cargo, but they lack the viral genes necessary for producing new infectious particles. This modification increases safety by preventing the spread of the viral vector beyond the initially transduced cells.

1 Introduction

1.1 Background

Gene therapy is a rapidly advancing medical treatment, showing significant progress, particularly in blood cancers and severe hereditary diseases. The growing number of approved gene therapies highlights their potential to treat diseases by modifying genes, altering their expression, or changing cell properties. Unlike treatments that typically provide temporary effects by targeting proteins, gene therapies can achieve long-lasting or curative outcomes through gene inhibition, addition, replacement, or editing.

Several gene therapies have already received approval in the EU, and clinical studies on these therapies have surged. These treatments primarily utilize viral vectors from lentiviruses, retroviruses, and adeno-associated viruses (AAV), which are genetically modified to directly introduce genes into patients or to modify patient cells outside the body before reintroduction.

In gene therapy, only somatic cells are altered. Modifying human germ cells, or germline modification, is prohibited as it would result in genetic changes that can be passed on to offspring. This type of genetic modification is regulated by the EU Commission and the European Medicines Agency (EMA), and is explicitly banned under the EU Clinical Trials Regulation[1]. Additionally, most Western European countries have laws in place to prevent intentional germline modification.

Despite the low estimated risk of unintended germline modification associated with viral vectors, genetically modified AAV vectors have been detected in some cases, including in humans. After administration, viral DNA can be found in semen, typically within seminal fluid or associated lymphocytes. To date, it has not been observed in sperm cells themselves; however, the absence of such findings cannot fully exclude the possibility of rare germline interactions. These viruses appear unable to cross the germline barrier, alleviating concerns about unintended germline modification.

Beyond these initial applications, new forms of gene therapy are emerging that involve the direct modification of the participant's genome, such as through CRISPR-Cas[2] systems. The first applications have already received regulatory approval in the UK, US, and EU. RNA-based therapies are also actively being explored. CRISPR and RNA-based therapies often bypass viral vectors, instead using alternative methods like liposomes, lipid nanoparticles, and polymers to deliver genetic material into cells. This approach helps to address challenges such as high production costs and the limited capacity of viral vectors to encapsulate long genetic sequences.

While natural physiological, immune, and anatomical barriers within the body significantly reduce the likelihood of gene therapy agents reaching and interacting with germline cells, concerns have been raised about the potential for non-viral gene delivery systems to cross the germline barrier. In the gonads, germ cells and somatic cells communicate through several mechanisms, including gap junctions, endocytosis, and extracellular vesicles, which transport nutrients, hormones, proteins, and small RNAs to germ cells. Because extracellular vesicles are capable of reaching germ cells it is theoretically possible that other nanoparticle-based carriers - such as lipid nanoparticles delivering a CRISPR-Cas construct - could also gain access. However, this is not the only conceivable pathway. Nanoparticle-based carriers might also reach germ cells indirectly through systemic biodistribution, where circulating particles penetrate reproductive tissues, or via

[1] Regulation (EU) No 536/2014 of the European Parliament and of the Council of 16 April 2014 on clinical trials on medicinal products for human use, and repealing Directive 2001/20/EC - OJ L 158, 27.5.2014, p. 1–76
[2] See "Glossary"

trafficking immune cells that have internalized genetic material and subsequently migrate into the gonads. In principle, such routes could enable unintended germline modification and therefore warrant careful consideration.

1.2 Purpose of this study

To strengthen the regulatory risk assessment, this project aimed to expand our understanding of the potential for unintended germline modification in emerging gene therapies, in order to better inform regulatory decision-making and guidance. This was achieved by systematically examining the following areas:

- Types of non-viral delivery systems for gene therapy,
- Data on potential biodistribution to gonadal tissue/germ cells,
- Current knowledge on transport across the germline barrier,
- Data on unintended germline modification in animal experiments,
- Indications of unintended germline modification from clinical studies.

In addition to analysing scientific literature and other sources of information on the potential for unintended germline modification, efforts were made to understand the regulatory requirements set by different authorities regarding germline transmission, particularly before proceeding to 'first in human' studies.

1.3 Scope of the study

The field of study is currently undergoing significant development. While some applications are advancing rapidly, others remain theoretical stage or pose a lower inherent risk of unintended germline modifications. Accordingly, the following research areas have been excluded from the scope of this report:

- *In vitro* intended germline modification;
- Cell therapy with *ex vivo* transduced cells.

The authors of this report acknowledge that the potential for unintended germline transformation raises significant ethical concerns. The absence of a detailed discussion on these ethical issues within the scope of this project should not be interpreted as a reflection of their lesser importance. On the contrary, such concerns underpin the broader prohibition of intentional germline modification and are therefore equally relevant in the context of unintended germline changes, even if these remain largely hypothetical. The authors anticipate that enhancing the robustness of the technical aspects of the risk assessment will contribute to a more comprehensive benefit-risk evaluation, thereby supporting well-informed ethical decision-making.

2 Overview of Delivery Systems for Gene Therapy

The method employed to deliver genetic material into target cells represents a pivotal factor influencing the overall efficacy of gene therapy. A wide variety of delivery systems have been developed, each with distinct mechanisms of action, performance characteristics, and clinical potential. In this section, these systems are grouped according to a mechanistic understanding of how they introduce payload into cells - through viral entry mechanisms, membrane fusion, receptor-mediated uptake, or physicochemical interactions.

Viral vectors, including AAV, lentiviruses, and adenoviruses, are among the most widely used systems in current clinical trials. Their success is largely due to their ability to efficiently enter cells and support sustained expression of the therapeutic gene. These viral vectors are engineered to be replication-deficient to ensure patient safety; nevertheless, they can still achieve a high therapeutic effect, as even a single viral particle may suffice to deliver its payload and trigger robust, and potentially long-lasting, gene expression. This high transduction efficiency enables a strong biological response from relatively small doses of viral vector material.

Non-viral delivery systems, such as lipid nanoparticles (LNPs), extracellular vesicles (EVs), liposomes, polymeric carriers, and peptide-based nanoparticles, differ from viral vectors in that they do not provide prolonged or self-sustaining gene expression; their therapeutic effect depends entirely on the initial dose delivered and the efficiency of uptake by target cells. Since the quantity of therapeutic payload is fixed at the time of delivery, gene expression typically diminishes over time. This characteristic makes non-viral systems well-suited for applications where transient or tightly controlled gene expression is desired. This is of particular importance when the payload consists of gene-editing components like CRISPR-Cas because sustained gene editor activity increases the risk of unintended DNA breaks and immune responses, compromising both safety and therapeutic efficacy.

The following section outlines the principal gene therapy delivery systems currently in use or under active investigation. **Table 1** provides a side-by-side comparison of the different system technologies, summarizing key properties such as clinical development status, transgene capacity, potential for genomic integration, immunogenicity, scalability, targeting strategies, *in vivo* behaviour, and their respective strengths and limitations.

2.1 Viral Vectors

Viral vectors are powerful delivery systems in gene therapy, designed to transport genetic material into target cells to treat or prevent diseases. At present, the three key viral vector strategies are based on adenoviruses, AAV, and lentiviruses each offering unique advantages in targeting specific tissues and ensuring long-term gene expression (1). Typically, a viral vector is defined by three key components:

1) the protein capsid and/or envelope, which encloses the genetic payload and determines the viral vector's tissue or cell tropism and antigen recognition;
2) the transgene of interest, which, when expressed in cells, confers the desired therapeutic effect;
3) the regulatory cassette, composed of enhancer, promoter, and auxiliary elements that control stable or transient transgene expression, either as an episome or chromosomal integrant.

Various platforms derived from these viral backbones have been engineered to improve delivery efficiency, reduce immunogenicity, and enhance therapeutic durability. For example, replication-

deficient AAV vectors, which are widely used in clinical gene therapy, can persist in target cells for extended periods as circular episomal DNA. Although they do not replicate, this long intracellular residence time results in sustained expression of the delivered genetic material, particularly in non-dividing cells (2). This persistence is an important consideration when evaluating potential off-target exposure of sensitive tissues such as the gonads and germline cells.

Similarly, gene editing strategies involving viral delivery of CRISPR-Cas components - especially when encoded within AAV vectors - can result in prolonged nuclease expression. Extended expression increases the cumulative duration of genome-editing activity, which may heighten the risk of unintended off-target genomic effects over time (3). These dynamics underscore the importance of carefully assessing exposure windows and expression kinetics in the design of safe and effective gene therapy products.

As of March 2023, there were over 3,900 gene therapy clinical trials worldwide, a number that has since risen past 4,000 (4). More than 70% of these use viral vectors, while non-viral delivery methods - though still a minority - are increasingly important in both preclinical and clinical settings (5, 6).

2.2 Viral-Like Particles (VLP)

Virus-like particles (VLPs) are nanoscale, non-replicative structures that mimic the architecture of viruses. Among the various viral sources for VLP construction, HIV-1-based VLPs have gained interest due to their design, functional versality and their ability to self-assemble with just one polyprotein (GAG) (7). They are generated by expression of the *gag* gene alone (Gag-VLPs) or in combination with the *env* gene (Gag-Env VLPs) yielding enveloped particles of approximately 150 nm in size that can incorporate both host-derived and engineered membrane proteins.

While VLP's accommodate both internal and surface modifications, enabling payload delivery and immunogenic applications, the formation of immature particles due to the absence of viral protease, or the potential host RNA encapsulation can pose limitations. Also, VLPs expressed *in vitro* need to be isolated from exosomes and microvesicles that are present in the cell supernatant, which may pose purification challenges and risks of immunogenic contaminants.

HIV-based VLPs have been explored as nanocarriers for CRISPR-Cas systems by fusing Cas9 to the GAG polyprotein and co-packing it with guide RNA (8). This Cas9-VLPs delivery tool of functional CRISPR-Cas9 ribonucleoproteins has been shown to be effective in primary human cells. This offers an outlook on VLPs becoming a relevant tool for targeted delivery of gene editing nucleases to somatic cells *in vivo*, although further research is necessary to optimize VLP production processes and to improve targeted delivery.

2.3 Lipid-based Nanoparticles

2.3.1 Extracellular Vesicles (EVs)

EVs which include exosomes and microvesicles, are cell-derived lipid bilayer structures that facilitate intercellular communication by carrying proteins, RNAs, and lipids. Their capacity to cross biological barriers, avoid rapid immune clearance, and deliver genetic material to target cells makes them a viable alternative to viral vectors (9). Compared to viral-based systems, EVs generally exhibit lower immunogenicity and cytotoxicity, which could be beneficial for applications requiring repeated administration.

Recent advancements in EV engineering have expanded their potential as gene delivery systems. Techniques such as donor cell modification, direct payload loading, and surface functionalization have improved their ability to carry and deliver specific nucleic acids, including mRNAs, siRNAs, and CRISPR-Cas components. While viral vectors remain the predominant platform in gene therapy, EV-based approaches are being actively explored due to their potential advantages in safety and payload versatility (10). Ongoing research aims to optimize EV production, purification, and scalability to facilitate their broader use in therapeutic applications.

2.3.2 Lipid Nanoparticles (LNPs)

LNPs represent a distinct class of lipid-based delivery systems, differing structurally and compositionally from conventional liposomes. While liposomes are characterized by an aqueous core that encapsulates hydrophilic molecules, LNPs typically feature a solid or structured lipid core in which nucleic acids are complexed within layered lipids (11).This core, often composed of ionizable lipids, stabilizes the payload and facilitates efficient cellular uptake and endosomal escape (12). The morphology of LNPs can vary depending on their lipid composition and formulation, allowing for tuneable properties that influence their biodistribution, stability, and transfection efficiency (13).

Advancements in LNP technology have been instrumental in enabling the clinical success of mRNA-based therapeutics, including vaccines and emerging gene therapy applications. LNP formulations typically include ionizable lipids, phospholipids, cholesterol, and polyethylene glycol (PEG)-conjugated lipids, each component contributing to stability, delivery efficiency, and immune evasion (14). Compared to traditional liposomes, LNPs offer superior protection of genetic material and more effective intracellular delivery, making them a promising platform for a broad range of gene-based treatments. LNPs currently represent the only non-viral delivery systems undergoing active investigation in human trials.

Table 2 provides an overview of gene editing agents delivered via LNPs that have progressed to clinical trials. The therapies listed span a range of genetic diseases, including hereditary transthyretin amyloidosis, hereditary angioedema, alpha-1 antitrypsin deficiency, hypercholesterolemia, and others.

A leading example is NTLA-2001, also known as Nexiguran ziclumeran (nex-z), developed by Intellia Therapeutics. It is the first CRISPR-Cas9-based *in vivo* gene-editing therapy to enter human trials. Targeting the *TTR* gene to treat hereditary transthyretin amyloidosis, NTLA-2001 is delivered intravenously via LNPs that encapsulate both mRNA encoding Cas9 and a guide RNA. Following promising results from a Phase I trial initiated in 2020, two global Phase III trials are now underway. Intellia's NTLA-2002 targets KLKB1 gene to treat hereditary angioedema. Early data from an ongoing Phase I/II trial suggest reduced disease burden. A global Phase III trial with NTLA-2002 began in 2025 following regulatory clearance. NTLA-3001, developed for alpha-1 antitrypsin deficiency, aimed to insert a functional SERPINA1 gene copy using CRISPR-Cas9 and LNP delivery. While regulatory approval was granted to initiate trials, the program was, according to communication by the developer, discontinued in early 2025 due to strategic prioritization.

Verve Therapeutics is advancing a pipeline of base-editing therapies (VERVE-101, -102, -201) targeting *PCSK9* and *ANGPTL3* for cardiovascular disease. VERVE-101 employs conventional LNPs, which naturally accumulate in the liver, while VERVE-102 and VERVE-201 use GalNAc-

LNPs for active hepatocyte targeting. These therapies are currently in Phase I trials across several countries.

YolTech Therapeutics has multiple ongoing early-phase trials, all using proprietary LNP delivery systems: YOLT-201 targeting the transthyretin gene in the liver to treat hereditary transthyretin amyloidosis, YOLT-203 targeting the HAO1 gene in the liver to treat primary hyperoxaluria type 1, and YOLT-204 editing the globin genes within hematopoietic stem cells to correct β-thalassemia.

Beam Therapeutics is testing BEAM-301 and BEAM-302, base-editing therapies targeting G6PC1 and SERPINA1, respectively. Both use LNPs and are in Phase I/II trials for metabolic and liver diseases.

CRISPR Therapeutics is developing CTX310 and CTX320, CRISPR-Cas9 therapies delivered via LNPs to target ANGPTL3 and Lp(a), respectively, for lipid-related cardiovascular conditions. Both are currently in Phase I development.

Finally, PBGENE-HBV by Precision BioSciences uses the ARCUS platform delivered by LNPs to target HBV DNA in hepatocytes. It received FDA Fast Track designation in 2025 and is in early clinical evaluation.

2.3.3 Liposomes

Liposomes are a subclass of lipid-based nanoparticles that differ structurally from LNPs, primarily in their morphology and lipid composition. These synthetic spherical vesicles are composed of one or more concentric lipid bilayers surrounding an aqueous core, enabling the encapsulation of both hydrophilic and hydrophobic molecules, including nucleic acids such as DNA, mRNA, and small interfering RNA (siRNA)(15). The ability of liposomes to encapsulate a wide variety of therapeutic payloads has made them a widely explored platform for gene delivery. In contrast to EVs, which are naturally secreted by cells and transport biomolecules such as proteins, lipids, and nucleic acids, liposomes are fully synthetic and can be engineered with precise control over size, surface charge, and lipid composition. This tunability allows optimization of stability, enhancement of cellular uptake, and improvement of targeted delivery to specific tissues.

Due to their biocompatibility and low immunogenicity, liposomes are considered a safer alternative to viral vectors, with a reduced risk of insertional mutagenesis (12). Various modifications, such as PEGylation to prolong circulation time or ligand conjugation for targeted delivery, have further enhanced their therapeutic potential. While liposome-based gene delivery systems continue to be refined, challenges such as stability, endosomal escape, and transfection efficiency remain key areas of focus for improving their clinical applicability. Ongoing research aims to optimize these properties to advance liposomes as a viable gene therapy platform.

2.4 Polymeric Systems

Polymeric systems are composed of synthetic or natural polymers, such as polyethyleneimine (PEI), poly(lactic-co-glycolic acid) (PLGA), chitosan, and poly(beta-amino esters) (PBAE), which interact with nucleic acids through electrostatic binding to form stable nanoscale complexes (16). Compared to LNPs, which encapsulate genetic material within a lipid core, polymeric systems offer a broad range of structural designs that can be optimized for enhanced gene delivery. Their

efficiency in cellular uptake is largely dependent on polymer composition, with some polymers, like PEI, exhibiting a proton sponge effect that facilitates endosomal escape.

While polymeric systems have been successfully employed in delivering plasmid DNA, siRNA, and CRISPR-Cas9 components, their clinical translation is often limited by challenges related to cytotoxicity and biodegradability (17). Modifications such as PEGylation and ligand conjugation have been explored to enhance stability, reduce toxicity, and improve targeted gene delivery (18). Despite these challenges, polymeric systems remain a promising alternative to viral and lipid-based delivery systems, with ongoing research focused on optimizing their properties for safe and efficient gene therapy applications.

2.5 Lipid-Free, Peptide-Based Nanoparticles (Pep-NP)

Peptide-based nanoparticles (Pep-NPs) are composed primarily of cationic peptides rich in basic amino acids such as lysine, arginine, and histidine. These nanoparticles form stable complexes with negatively charged nucleic acids through electrostatic interactions. This interaction not only condenses genetic material, reducing its size, but also provides protection against enzymatic degradation. The structural flexibility of Pep-NPs enables the encapsulation of various nucleic acids, including plasmid DNA, mRNA, siRNA, and antisense oligonucleotides, making them suitable for a wide range of therapeutic applications (19).

Additionally, incorporating cell-penetrating peptides (CPPs) - short peptides (6–30 amino acids) capable of transporting diverse payload across cell membranes while maintaining their functional integrity - enhances cellular uptake and facilitates efficient intracellular delivery of genetic material (20). Recent advancements have focused on functional modifications, such as adding targeting sequences or PEGylation, to enhance specificity and prolong circulation time, further improving their therapeutic efficacy potential (21). Overall, Pep-NPs offer a versatile and efficient platform for non-viral gene delivery, with ongoing research dedicated to optimizing their design for clinical applications as challenges remain such as limited stability, suboptimal delivery efficiency, scalability challenges etc (22).

2.6 Gold Nanoparticles (AuNP)

Gold nanoparticles (AuNPs) can be functionalized with DNA, RNA, or CRISPR-Cas9 components, allowing for targeted gene regulation and genome editing. Their small size and customizable surface chemistry enable efficient cellular uptake, and modifications such as ligand conjugation improve targeting and transfection efficiency. AuNPs have been employed in delivering RNA-based therapeutics, as well as CRISPR-Cas9 ribonucleoproteins for precise gene-editing applications (23). However, research in AuNPs mediated gene therapy is still at the preclinical stage. Despite their potential, challenges remain in optimizing endosomal escape and minimizing off-target effects, both in terms of non-specific tissue uptake and unintended genomic alterations. Continued research aims to refine these systems for improved gene delivery and clinical translation.

2.7 Magnetic Nanoparticles (MNP)

Magnetic nanoparticles (MNPs) can be directed to specific target cells or tissues using external magnetic fields by attaching therapeutic genes to biocompatible MNPs, enhancing transfection efficiency. This method, known as magnetofection, offers rapid and effective gene transfer, as

demonstrated in various studies (24, 25). MNPs can be engineered with functional coatings such as PEI or other biocompatible polymers to improve nucleic acid binding, cellular uptake, and endosomal escape, further enhancing gene transfection efficiency (26). Additionally, their ability to be guided using magnetic fields allows for localized gene delivery, reducing off-target tissue effects and increasing therapeutic precision.

MNP-based systems have shown promise for delivering plasmid DNA, siRNA, and CRISPR-Cas9 components in preclinical studies (27), making them a versatile tool for gene therapy applications. However, challenges such as optimizing nanoparticle stability, biodegradability, and ensuring minimal cytotoxicity remain key areas of ongoing research to enhance their clinical applicability (26).

2.8 Proteolipid Vehicles

Proteolipid vehicles are hybrid nanoparticles composed of lipid components that improve biocompatibility and facilitate membrane fusion, along with peptide or protein elements that enhance nucleic acid binding and cellular targeting. A key feature of this system is the incorporation of fusion-associated small transmembrane (FAST) proteins derived from the non-enveloped fusogenic orthoreovirus (28). By combining the FAST protein with a well-tolerated lipid formulation, efficient nucleic acid delivery across various tissues can be achieved, as demonstrated in ocular gene delivery using the FAST-PLV platform (29). The FAST protein enables proteolipid vehicles (PLVs) to fuse directly with the plasma membrane of target cells, enhancing transfection efficiency. In comparison to conventional LNP formulations and PLVs lacking FAST proteins, the 41N FAST-PLV platform exhibited significantly improved DNA and RNA delivery *in vitro*, highlighting the superior performance of this hybrid system (29).

Key messages

- While a wide diversity of viral and non-viral gene delivery systems is being developed, only **few** have reached an advanced **stage of clinical development**.
- Viral vectors ensure long-term gene-expression and have reached marketing authorization for *in vivo* and *ex vivo* gene therapy applications,
- **LNP** are increasingly being explored and utilized as delivery system for gene editing components because of their **transient delivery**, lower concerns for off-target effects - particularly non-specific tissue uptake - and lower manufacturing costs.
- **Except for liposomes and VLPs, other non-viral platforms** did **not (yet) reach clinical investigation** but ongoing efforts to increase their target versability, purification and scalability may soon facilitate their clinical translation / applicability.

Table 1. Comparative overview of gene therapy delivery systems. *This table aims to provide a simplified comparison of gene therapy delivery systems, focusing on their underlying mechanisms, technical characteristics, and translational potential. It summarizes key characteristics of major viral and non-viral gene delivery systems. Each system is evaluated in terms of its clinical use, payload capacity, potential for genomic integration, immunogenicity, scalability, targeting strategies, and whether it demonstrates in vivo amplification or functional persistence. The final columns outline the strengths and limitations of each approach.*

Delivery system	*In vivo* amplification or expansion	Clinical use	Transgene capacity	Integration into host genome	Composition / Structural components	Scalability	Targeting strategies	Monitoring techniques	Strengths	Limitations
Adeno-associated virus (AAV)	No	Yes	~4.7 kb	Mostly episomal	Protein capsid with single-stranded DNA	Moderate	Tissue tropism Capsid engineering	qPCR / ddPCR PET and SPECT	Long-term expression Low pathogenicity	Limited payload Pre-existing immunity
Lentivirus	No	Yes	~8 kb	Integrating	Enveloped virus (lipid membrane + glycoproteins) with RNA genome	Moderate	Pseudotyping Promotor targeting	qPCR/ ddPCR BLI	Stable integration Works in dividing/non-dividing cells	Insertional mutagenesis risk High immunogenicity
Adenovirus	No	Yes	Up to ~36 kb	Episomal	Non-enveloped protein capsid Double-stranded DNA genome	High	Promotor engineering	qPCR/ddPCR ISH IHC	Large payload High expression	High immune response Short-lived expression
Viral-like particles (VLPs)	No	Yes	~5-10 kb	Non-integrating	Protein-based capsid-like structures without viral genome	Moderate	Surface engineering	TEM (30, 31) Fluorescence imaging (32) MRI (33) PET Tunable resistive pulse sensing (32) Flow virometry (32)	Safer mimic of viral delivery	Complex production Lower efficiency

Delivery system	*In vivo* amplification or expansion	Clinical use	Transgene capacity	Integration into host genome	Composition / Structural components	Scalability	Targeting strategies	Monitoring techniques	Strengths	Limitations
Lipid Nanoparticles (LNPs)	No	Yes	Flexible	Non-integrating	Ionizable lipids Helper lipids (cholesterol, phospholipids) PEG-lipids	High	Ionizable lipids PEG GalNAc	TEM (34) LSC (35) Fluorescence Imaging (34, 36) BLI (37) CT (35) MRI (35) PET and SPECT (35)	Proven in mRNA vaccines High liver delivery	Toxicity Reactogenicity
Extracellular vesicles (EVs)	No	No	~5-10 kb	Non-integrating	Natural lipid bilayer vesicles Membrane protein Endogenous payload	Moderate	Donor-cell tropism Surface ligands	Fluorescence Imaging (38) BLI (38) CT (38) MRI (38) PET and SPECT (38)	Low toxicity	Heterogeneous Difficult to scale
Liposomes	No	Yes	Flexible	Non-integrating	Phospholipid bilayer Optional with PEG Aqueous interior	Moderate	Size Charge Surface ligands	Fluorescence imaging (39) MicroSPECT/CT imaging (40)	Safe Modifiable	Poor endosomal escape Lower efficiency
Polymeric Systems	No	No	Flexible	Non-integrating	Cationic polymers Optional with PEG	Moderate-High	PEGylation Chemical targeting	No data	Customizable Biodegradable option	Cytotoxicity (some) Less efficient *in vivo* Non-degradable (some)
Peptide-based nanoparticles (PeP-NP)	No	No	Flexible	Non-integrating	Cationic peptides Optional with CPPs or PEG	Moderate	Cell-penetrating peptides Targeting motifs	Fluorescence Imaging (41) PET and SPECT (42)	High uptake Easy to engineer	Limited stability Suboptimal delivery efficiency Scalability challenges

Delivery system	*In vivo* amplification or expansion	Clinical use	Transgene capacity	Integration into host genome	Composition / Structural components	Scalability	Targeting strategies	Monitoring techniques	Strengths	Limitations
Gold nanoparticles (AuNPs)	No	No	Flexible	Non-integrating	Gold core with thiol-linked DNA/RNA Optional CPPs or PEG	High	Surface modification	TEM (43) MRI (44) PET (45)	Small size Surface modifiable	Limited endosomal escape Non-degradable
Magnetic nanoparticles (MNPs)	No	No	Flexible	Non-integrating	Iron oxide core with polymer or lipid coating Often PEI-functionalized	Moderate	Magnetically guided	No data	Targeted via magnetic field	Non-degradable
Proteolipid vehicles (PLVs)	No	No	Flexible	Non-integrating	Lipid membrane FAST proteins Peptide or protein targeting elements	Moderate	FAST proteins Membrane fusion	No data	Enhanced fusion ability Hybrid design High payload	Suboptimal tissue-specific

Table 2. Clinical-stage gene editing therapies delivered via lipid nanoparticles (LNPs). *This table provides an overview of LNP-enabled gene editing therapies that have entered clinical development. Each entry includes the drug name, sponsor, gene editing platform (e.g., CRISPR/Cas9 or base editing), target gene, clinical phase, and details such as the number of participants, therapeutic indication, trial identification number (NCT), route of administration, study timeline, and geographic trial locations.*

Name of the Drug	Company	System	Target gene	Phase	Patients number	Conditions	NCT	Route of administration	Date	Location	Ref.
NTLA-2001	Intellia Therapeutics	CRISPR-Cas9 gene-editing therapy	TTR	I	72	Hereditary transthyretin amyloidosis (hATTR)	NCT04601051	Intravenous	2020-2026	FR, GB, NZ, SE	[3]
TLA-2001	Intellia Therapeutics	CRISPR-Cas9 gene-editing therapy	TTR	III	765	Transthyretin Amyloidosis with Cardiomyopathy (ATTR-CM)	NCT06128629	Intravenous	2023-2028	AR, AU, CA, DE, DK, ES, FR, GB, HU, IL, IT, KR, NL, NZ, PT, SE, SG, TW, US	[4] [5]
NTLA-2001	Intellia Therapeutics	CRISPR-Cas9 gene-editing therapy	TTR	III	50	Hereditary Transthyretin Amyloidosis with Poly-neuropathy (ATTR-PN)	NCT06672237	Intravenous	2024-2028	AU, NZ	[6] [7]
NTLA-2002	Intellia Therapeutics	CRISPR-Cas9 gene-editing therapy	KLKB1	I, II	37	Hereditary Angioedema (HAE) = inflammatory disease	NCT05120830	Intravenous	2021-2026 Temporary on hold	AU, FR, GB, DE, NL, NZ	[8]
NTLA-2002	Intellia Therapeutics	CRISPR-Cas9 gene-editing therapy	KLKB1	III	60	Hereditary Angioedema (HAE)	NCT06634420	Intravenous	2025-2027	CA, GB, US	[9]
NTLA-3001	Intellia Therapeutics	CRISPR-Cas9 gene-editing therapy	AAT	I/II	30	Lung disease associated with alpha-1 antitrypsin deficiency (AATD)	NCT06622668	Intravenous	WITHDRAWN	N/A	[10]

[3] https://clinicaltrials.gov/study/NCT04601051 - accessed on 03 July 2025

[4] https://www.intelliatx.com/ - accessed on 19 May 2025

[5] https://clinicaltrials.gov/study/NCT06128629?term=NTLA-2001&rank=4 – accessed on 19 May 2025

[6] https://www.intelliatx.com/ - accessed on 19 May 2025

[7] https://clinicaltrials.gov/study/NCT06672237?term=NTLA-2001&rank=2 - accessed on 19 May 2025

[8] https://clinicaltrials.gov/study/NCT05120830 - accessed on 03 July 2025

[9] https://clinicaltrials.gov/study/NCT05120830?term=NTLA-2002&rank=1 - accessed on 19 May 2025

[10] https://clinicaltrials.gov/study/NCT06622668?term=NTLA-3001&rank=1 - accessed on 19 May 2025

Name of the Drug	Company	System	Target gene	Phase	Patients number	Conditions	NCT	Route of administration	Date	Location	Ref.
VERVE-101	Verve Therapeutics, Inc.	Base-editing therapy	PCSK9	Ib	44	Heterozygous familial hypercholesterolemia, artherosclerotic cardiovascular disease	NCT05398029	Intravenous	2022-2025 Temporary on hold	GB, NZ	[11]
VERVE-102	Verve Therapeutics, Inc.	Base-editing therapy	PCSK9	Ib	36	Heterozygous Familial Hypercholesterolemia or Premature Coronary Artery Disease	NCT06164730	Intravenous	2024-2027	AU, CA, GB, NZ	[12]
VERVE-201	Verve Therapeutics, Inc.	Base-editing therapy	ANGPTL3	I	36	Refractory Hypercholesterolemia	NCT06451770	Intravenous	2024-2027	CA, GB	[13]
YOLT-201	RenJi Hospital	CRISPR-Cas9-based gene-editing therapy	TTR	I	7	ATTR-CM	NCT06082050		2023-2026	CN	[14]
YOLT-201	YolTech Therapeutics	CRISPR-Cas9-based gene-editing therapy	TTR	I, IIa	31	ATTR-PN ATTR-CM	NCT06539208	Intravenous	2024-2026	CN	[15]
YOLT-203	RenJi Hospital	CRISPR-Cas9-based gene-editing therapy	HAO1	I	7	Primary Hyperoxaluria Type 1 (PH1)	NCT06511349	Intravenous	2024-2026	CN	[16]
YOLT-204	YolTech Therapeutics	CRISPR-Cas9-based gene-editing therapy	Regulatory region of hemoglobin	I	3	transfusion-dependent β-thalassemia	NCT06678165	Intravenous	2025-2026	CN	[17]
BEAM-301	Beam Therapeutics	Base-editing therapy	G6PC1	I, II	36	Glycogen storage disease 1a (GSD 1a)	NCT06735755	Intravenous	2024-2027	US	[18]

[11] https://clinicaltrials.gov/study/NCT05398029 - accessed on 03 July 2025

[12] https://clinicaltrials.gov/study/NCT06164730 - accessed on 03 July 2025

[13] https://www.genengnews.com/topics/genome-editing/bittersweet-symphony-verves-pause-on-verve-101-narrows-lnp-delivery-strategy/ - accessed on 19 May 2025

[14] https://clinicaltrials.gov/study/NCT06082050?term=YOLT-201&rank=2 - accessed on 19 May 2025

[15] https://clinicaltrials.gov/study/NCT06539208?term=YOLT-201&rank=1 - accessed on 19 May 2025

[16] https://clinicaltrials.gov/study/NCT06511349?term=YOLT-203&rank=2 - accessed on 19 May 2025

[17] https://www.biospace.com/press-releases/yoltech-therapeutics-to-initiate-a-clinical-trial-for-yolt-204-a-first-in-class-bone-marrow-targeted-in-vivo-gene-editing-therapy-for-%CE%B2-thalassemia - accessed on 19 May 2025

[18] https://clinicaltrials.gov/study/NCT06735755?term=BEAM-301&rank=1 - accessed on 19 May 2025

Name of the Drug	Company	System	Target gene	Phase	Patients number	Conditions	NCT	Route of administration	Date	Location	Ref.
BEAM-302	Beam Therapeutics	Base-editing therapy	SERPINA1	I, II	106	Alpha-1 antitrypsin deficiency (AATD)	NCT06389877	Intravenous	2024-2027	AU, GB, NL, NZ	[19]
CTX310	CRISPR Therapeutics	CRISPR-Cas9 gene-editing therapy	ANGPTL3	I		Dyslipidaemia	Not data	Intravenous	No data	No data	[20] [21]
CTX320	CRISPR Therapeutics	CRISPR-Cas9 gene-editing therapy	Lp(a)	I		Atherosclerotic cardiovascular disease (ASCVD)	Not data	Intravenous	No data	No data	[22]
PBGENE-HBV	Precision BioSciences	ARCUS gene-editing	N/A	I	45	Chronic Hepatitis B	NCT06680232	Intravenous	2024-2026	MD, HK, NZ	[23]

AR, Argentina; AU, Australia; CA, Canada; CN, China; DE, Germany; DK, Denmark; ES, Spain; FR, France; GB, United Kingdom; HK, Hong Kong; HU, Hungary; IL, Israel; IT, Italy; KR, South Korea; MD, Moldova; NL, Netherlands; NZ, New Zealand; PT, Portugal; SE, Sweden; SG, Singapore; TW, Taiwan; US, United States ; Note: “No data” indicates that, to date, no publicly available data have been reported for that parameter.

[19] https://clinicaltrials.gov/study/NCT06389877?term=BEAM-302&rank=1 - accessed on 19 May 2025

[20] https://ir.crisprtx.com/static-files/e5359024-937f-444f-96da-1d27fd0f9361 - accessed on 19 May 2025

[21] https://innovativegenomics.org/news/crispr-clinical-trials-2024/ - accessed on 19 May 2025

[22] http://pharmaceutical-technology.com/data-insights/ctx-320-crispr-therapeutics-atherosclerosis-likelihood-of-approval/ - accessed on 19 May 2025

[23] http://clinicaltrials.gov/study/NCT06680232?term=PBGENE-HBV%20&rank=1 – accessed on 19 May 2025

3 Biodistribution of Delivery Systems

3.1 General Biodistribution Patterns

Understanding the biodistribution of viral and non-viral gene delivery systems is fundamental to evaluating both the safety and therapeutic potential of gene therapies. Biodistribution determines which tissues or organs are exposed to the therapeutic agents and thus underpins any subsequent consideration of off-target tissue effects, particularly unintended exposure of gonadal tissue. In the absence of biodistribution to gonadal tissues, no biological interaction can occur, thereby eliminating the potential risk of germline modification. Broad systemic biodistribution can be advantageous for treating conditions that require widespread delivery, such as muscular dystrophies or metabolic storage disorders; however, it also heightens the risk of unintended exposure in non-target tissues. Conversely, highly targeted delivery systems may improve safety by limiting off-target distribution, though this specificity could constrain their applicability across diverse clinical indications.

Several factors influence the distribution of delivery systems in the body, including the dose administered and the route of administration (*e.g.*, systemic *vs.* localized). Also, physicochemical properties of the delivery system play a critical role. Key factors include:

- Particle size
  - Smaller particles (<200 nm) can more easily navigate through biological barriers and reach target tissues.
- Surface charge
  - Positively charged delivery systems typically show enhanced cellular uptake but may interact non-specifically with other tissues, increasing off-target accumulation.
- Surface modifications
  - Functionalization's such as PEGylation can improve circulation time by reducing immune recognition and clearance.
- Lipid composition
  - In LNPs, transfection efficiency is influenced by the structure and geometry of cationic lipids, the nature of lipid anchors, and linker bonds. Even subtle changes in lipid architecture can drastically alter *in vivo* behaviour, often leading to preferential accumulation in organs like the liver.

Gene delivery systems can be engineered with peptide ligands or other targeting molecules to enhance tissue-specific uptake. This strategy facilitates the directed transport of therapeutic agents to intended tissues while minimizing accumulation in non-target regions. Incorporating such specificity into delivery systems design is an important approach to optimizing therapeutic efficacy and reducing systemic exposure.

Systemically administered delivery systems - whether CRISPR-Cas9 systems, viral vectors, or nanoparticles - face numerous barriers in reaching gonadal tissues:

- Rapid clearance by the liver, spleen, and kidneys;
- Enzymatic degradation and immune neutralization;
- Preferential uptake by non-target organs (e.g. liver, lungs, muscle);
- Endosomal entrapment, particularly for non-viral delivery systems, limiting cytosolic delivery.

Natural physiological, immune, and anatomical barriers within the body significantly reduce the likelihood of gene therapy agents reaching and interacting with germline cells. Nevertheless, biodistribution data remain essential for evaluating this risk. Since biological effects require direct exposure, understanding the distribution of therapeutic agents - including potential access to gonadal tissues - is critical for conducting a comprehensive safety assessment.

3.2 Biological Barriers in the Male Reproductive System

Figure 1 presents a schematic overview of the male reproductive system, highlighting the spatial arrangement of various cell types and tissue layers that may act as biological barriers to unintended germline modification.

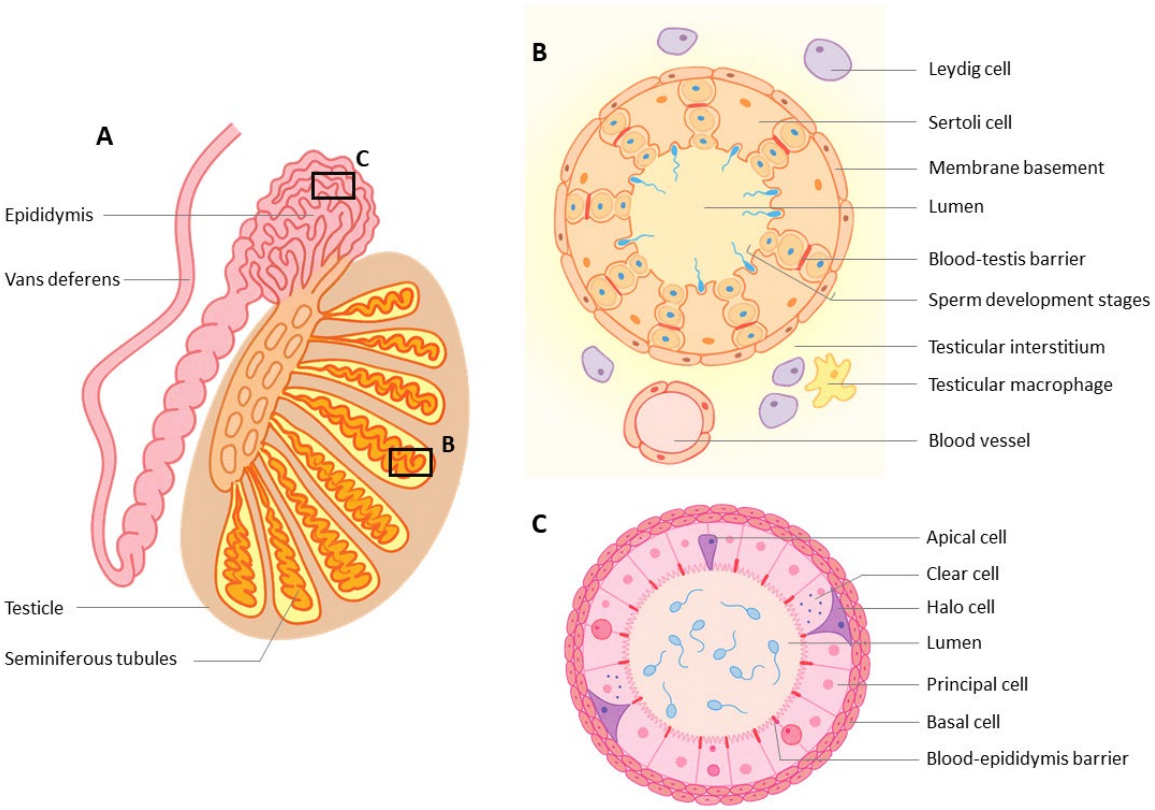

***Figure 1. Schematic representation of the male reproductive system. A**. Anatomy of the testicle: The testes are mainly composed by seminiferous tubules. Once produced in the seminiferous tubules, developing spermatozoa are transported through the lumen of the seminiferous tubules to the epididymis where they are stored. From the epididymis, it moves into the vas deferens where it is mixed with seminal fluid to form semen. **B**. Traverse section of a seminiferous tubule and the testicular interstitium. The interstitium is composed of blood vessels, Leydig cells, testicular macrophages, and other immune cells. Blood-borne agents capable of crossing the vascular endothelium surrounding the blood vessels, enters the interstitial space where an inflammatory response can be activated, inducing the recruitment and activation of immune cells. Sertoli cells provide a safe and nutritional environment for spermatogenesis. The Blood-testis barrier prevents harmful substances, immune cells or antibodies from entering the luminal compartment of the seminiferous tubules. **C**. A traverse section of the epididymis. The epididymis, a long, coiled tube protects sperm from the external environment during their maturation, transport and storage. The blood-epididymis barrier maintains a specialized environment for sperm maturation by restricting molecular movement between the blood and the epididymal lumen.*

The barriers in both the testis and epididymis involve a complex interaction between epithelial cell tight junctions (the physical or anatomical barrier), physiological components (transporters, channels at the basolateral and apical membranes, and the paracellular route) and immunological components (inside and outside the tubule/duct)(46).

A relative broad range of wild-type viruses reach gonadal tissues via hematogenous spread and are known to bypass biological barriers in the male reproductive systems. Key mechanisms involve exploiting structural vulnerabilities, in particular at the Blood-Testis-Barrier (BTB), immune evasion strategies or cell tropism (**Table 3**). An understanding of the mechanism underlying natural viral infection of the male reproductive system may provide insights on the risk factors for delivery systems for gene therapy to inadvertently penetrate, bypass biological barriers or to be sexually transmitted.

Another area of research that may help to understand mechanistic features enabling the crossing of biological barriers from the male reproductive system is the field of preclinical studies aiming at restoring male infertility. Lentiviral, adenoviral and adeno-associated viral vectors appear to be the most exploited delivery systems used in experimental mice studies in this field of research while several non-viral delivery systems such as Sertoli cell-derived exosomes or extracellular vesicles have been shown to cross the BTB and to reach spermatogonial stem cells. Although the BTB may serve as a biological shield that limits the penetration of certain components to spermatogonial cells, it is important to recognize that spermatogonia undergo continuous cell division after puberty. This ongoing proliferative activity may increase their susceptibility to unintended genetic alterations, either due to insufficient DNA repair mechanisms during rapid cell division or off-target genomic effects caused by gene-editing components.

Table 3. Biological barriers in the male reproductive system. *(The references are indicative of key mechanisms and challenges in overcoming the biological barriers of the male reproductive system and are not intended as an exhaustive review of the literature.)*

Structure/ tissue/ cells	Location	Function	Viral entry mechanisms	Viral delivery systems for preclinical studies examining gene therapies restoring male fertility	Non-viral delivery systems for preclinical studies examining therapies restoring male fertility
Vascular endothelium	Surrounding the blood vessels	First barrier of epithelial cells against blood-borne agents preventing entry into testes	• Flaviviruses' non-structural proteins cause endothelial dysfunction and vascular leak (47)	No viral delivery systems reported.	No non-viral delivery approaches reported.
Testicular interstitium	Surrounds seminiferous tubules	Microenvironment for germ cells with blood, lymphatic vessels, LCs, immune cells	• HIV-infected CD4+ macrophages migrate into the testicular interstitium and travers the BTB upon a temporary breach ('Trojan horse')(48)	No viral delivery systems reported.	No non-viral delivery approaches reported.
Leydig cells (LC)	Interstitial space between tubules	Testosterone production and secretion; Supports SC	• ACE2 and TMPRSS2 proteins expressed in LCs assist SARS-CoV-2 cell entry and priming into cells (49, 50) • LC and SC sialic acid mediate MuV virus entry and inflammation inducing disruption of BTB integrity and permeability (51)	• Targeted by AAV8 and AAV-DJ upon testicular interstitial injection (mice). Neither germ cells nor SC are target cell of AAV8 (mice, NHP)(52, 53).	• In a *in vivo* rat model, SC derived exosomes reach ST and interstitial space after injection in efferent duct, suggesting BTB crossing (54)
Seminiferous tubules (ST)	Coiled structures in the testis	Spermatogenesis; The basement membrane acts as a physical barrier	No evidence of viral entry reported.	No viral delivery systems reported.	No non-viral delivery approaches reported.
Spermatogonial stem cells (SSC)	Basal compartment of ST	Lifelong production of sperm	No evidence of viral entry reported.	• AdV vectors transduce SSCs, with no germline integration in offspring (55) • *In vivo* microinjected LV vectors penetrate the basement membrane and the BTB, transducing SSC and testicular somatic cells (56)	• Exosomes (57), SC-EVs (54), LNPs (58), ferritin nanocarriers (59) deliver to SSCs with no germline integration • Fibroin nanoparticle-encapsulated cationic lipid complex (Fibroplex) upon intratesticular injection in mice (60)
Blood-testis barrier (BTB)	Between SCs, near the basement membrane of ST	Barrier of epithelial cells interconnected by tight junctions hampering access to ST	• ZIKV (61, 62), HIV (61), EBOV (61), MARV (63) use cytoskeletal rearrangement, immune cell recruitment, and altered tight junction protein expression to compromise BTB integrity during periodic BTB restructuring and weakening of spermatogenis	• AAVs, directly injected into the ST, cross the BTB and the basement membrane, reaching the germ cells and SCs (64)	• SC-Evs loaded with miR-24-3p inhibitors cross BTB (65) • Nanoparticles (CAP LNPs) deliver mRNA and saRNA in spermatocytes (58)

Sertoli cells (SC)	Within the ST	Structural and nutritional support to germ cells	• SARS-CoV-2 cell entry via ACE2 and TMPRSS2 proteins expressed in SCs (49, 50) • MuV entry into SCs, via sialic acid and AXL- and MER-RTK binding, triggering cytokines release, disrupting key tight junction proteins, weakening the BTB and reaching testicular lumen (61, 66) • ZIKV triggers AXL-RTK mediated infection and SC entry by TNF-α release, inducing BTB permeability weakening (66, 67)	• Long term transgene expression in mice, without germline integration, upon LV vector transduced SCs(68) or microinjected AdV vectors into ST(69)	• SC-EVs deliver miRNA inhibitors; fibroin nanoparticles show testicular delivery (70)
Epididymis	Coiled tube on posterior of testis	Sperm maturation and storage	• Epididymal lesions due to ZIKV infection cause inflammation and subsequent, disruption of tissue structure and tight junction via IL-6/IL-28 (71)	No viral delivery systems reported.	No non-viral delivery approaches reported.
Blood-Epididymis Barrier (BEB)	Between blood vessels and epididymis	Barrier of epithelial cells interconnected by tight junctions preventing access to the epididymal lumen and protecting sperm	• HIV, ZIKV and HPV have been found to be present in the epididymis (72)	No viral delivery systems reported.	No non-viral delivery approaches reported.
Epididymal Microenviron-ment	Within the epididymis	Acidic pH of 6,5 to support the final stages of sperm development, storage, and protection	No evidence of viral entry reported.	No viral delivery systems reported.	No non-viral delivery approaches reported.
Sperm Membranes	On spermatozoa in the testes and epididymis	Sperm protection and fertilization	• ZIKV binds Tyro3 receptor at spermatozoa mid-piece (73) • HSV-2 internalizes into spermatozoa (74)	No viral delivery systems reported.	• Liposomes improve DNA uptake in sperm in chicken and rabbit (70)
Seminal fluid	Secreted by accessory glands	Sperm nourishment and motility	• Diverse viruses in semen due to replication ability, immune evasion, viremia, structural stability, and barrier permeability changes (75)	No viral delivery systems reported.	No non-viral delivery approaches reported.

AAV, adeno-associated virus; ACE2, angiotensin I-converting enzyme 2; AdV, Adenovirus; BTB, Blood-Testis Barrier; DNA, Deoxyribonucleic Acid; EBOV, Ebola virus; HIV, Human immunodeficiency virus; HSV, Herpes simplex virus; IL, Interleukine; LC, Leydig cells; LNP, Lipid nanoparticle; LV, Lentivirus; MARV, Marburg virus; miR-24-3p, microRNA; mRNA, messenger ribonucleic acid; MuV, mump virus; NHP, non-human primate; RTK, receptor tyrosine kinases; saRNA, self-amplifying RNA; SARS-CoV-2, Severe acute respiratory syndrome coronavirus 2; SC, Sertoli cells; SC-EV, Sertoli cell-derived extracellular vesicles; SSC, spermatogonial stem cells; ST, Seminiferous tubules; TMPRSS2, transmembrane serine protease 2; TNF, tumor necrosis factor; ZIKV, Zika virus ; Note: “No evidence of viral entry reported” or “No (non)-viral delivery systems reported” means that, to date, no published or publicly available data describing viral or non-viral delivery approaches targeting this structure or cell type in preclinical models have been identified.

3.3 Biological Barriers in the Female Reproductive System

Figure 2 presents a schematic overview of the female reproductive system. The female reproductive tract comprises distinct anatomical and cellular components that present specific barriers for harmful agents, compounds or drugs.

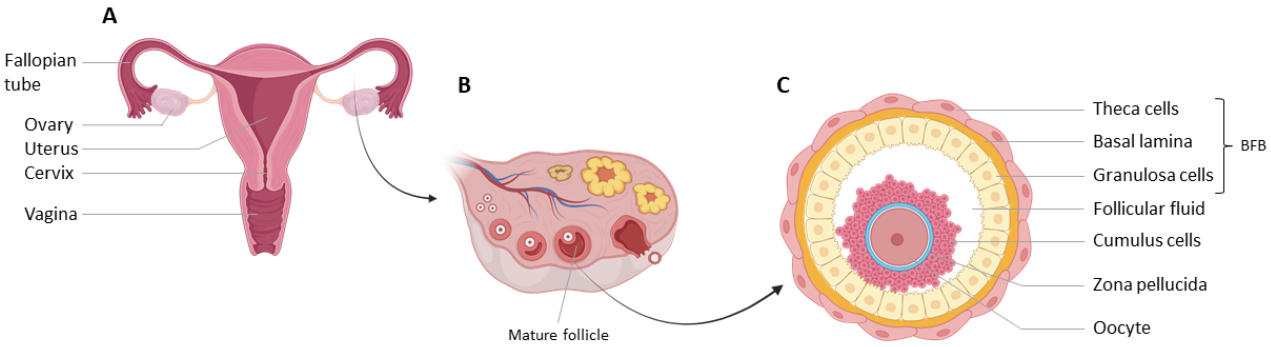

***Figure 2. Female reproductive system** : **A**. The internal organs of the female reproductive system. **B.** Ovary showing the follicles at different developmental stages: Primary follicles; secondary follicles, antral follicle, mature follicle, ovulation, corpus luteum and corpus albicans. **C**. Structure of a mature ovarian follicle. The zona pellucida, the first barrier, is an extracellular layer immediately surrounding the oocyte. The cumulus cells act as a second biological barrier protecting the oocyte from harmful substances in the surrounding environment. The blood-follicle barrier (BFB) acts as a selective filter by controlling the movement of molecules based on size and charge. The BFB is composed of different layers of cells: the granulosa cells playing a key role in the development and growth of ovarian follicles, the basal lamina providing structural support to the follicle and serving as a selective barrier regulating the passage of nutrients, hormones, and other substances between the follicle and the surrounding ovarian stroma and the theca cells on its outer surface supporting growth and maturation of ovarian follicles. Created with BioRender.com*

Epithelial cells lining the lower reproductive tract and the cervical mucus act as primary physical and biochemical barrier. Within the ovary, additional selective barriers exist, including the blood-follicle barrier (BFB), which regulates access to the follicular environment. Critical intrafollicular targets such as theca cells, granulosa cells, the zona pellucida, and oocytes are tightly protected, by various physical and cellular barriers to maintain their structural integrity and facilitate communication, requiring therefore advanced strategies to overcome these biological barriers (**Table 4**). In addition, contrary to the continuous proliferative activity of spermatogonial cells, oocytes remain quiescent in meiotic arrest from foetal life until ovulation. This relatively short time for cell division may render oocytes less prone to unintended errors during replication or even off-target genomic effects from gene editing as compared to the continuously proliferating spermatogonial cells.

Several studies suggest the ability of specific viruses to cross the BFB (76, 77), however, to the best of our knowledge, no direct evidence for this has been delivered. Findings also indicate that inflammatory conditions may alter the permselectivity of the BFB, potentially facilitating viral entry during systemic infections (78).

Some studies highlight the potential of engineered viral vectors to overcome biological barriers in female reproductive system therapeutics. There is direct evidence of penetration of the BFB by AAV9 upon direct injection into the ovarian stroma in a mouse model, presumably by

transcytosis. Importantly, while no transgenic DNA could be found in the offspring, study limitations preclude making definite conclusion on viral vector integration into the oocytes (77).

Oncolytic viral vectors such as engineered adenoviruses are also studied for their potential in malignant and benign gynaecological disorders, however, to the best of our knowledge, no BFB crossing was explicitly demonstrated for these viral vectors (79)

An emerging field of research aims at identifying the potential of CRISPR-Cas technology in gynaecological cancers. For example, antibody-conjugated LNP harbouring CRISPR-Cas components were deployed in an ovarian cancer mouse model, resulting in a high yield of targeted gene-editing of ovarian tumour cells (80). This showed the potential of targeted LNP to overcome the main off-target tissue of LNP-platforms namely the liver. A biodistribution study on mice with 80 nm LNP showed that these LNPs penetrate into the ovarian cells but are restricted on the basal membrane and in the thecal layer, suggesting their inability to cross the BFB (81).

Studies on follicular development and disease models also indicate the capacity of follicular-fluid-derived extracellular vesicles to cross the follicular environment (78, 82). However, direct *in vivo* studies confirming their traversal from the bloodstream into follicles remain scarce. Advanced labelling and biodistribution assays may further shed light on their barrier-crossing mechanism. The engineering of extracellular vesicles for therapeutic purposes is being explored, however studies did not reach clinical research yet.

This evaluation is intentionally limited to scenarios in which unintended germline modification may occur as a result of administration of gene therapy to the individual. While speculative scenarios - such as the potential transmission of gene therapy delivery systems through sexual contact to an untreated partner - could theoretically raise additional concerns, these fall outside the current scope. Such possibilities may warrant further investigation as gene therapy applications become more widespread. It is important to note that clinical gene therapy trials routinely implement precautionary measures, including the prevention of pregnancy and the use of barrier contraception, to mitigate any risk of germline exposure.

Table 4. Biological barriers to the female reproductive system *(The references are indicative of key mechanisms and challenges in overcoming the biological barriers of the female reproductive system and are not intended as an exhaustive review of the literature.).*

Structure/ Region	Location	Function	Known viral infection mechanism	Viral delivery systems for preclinical studies examining gene therapies restoring female fertility or treating reproductive diseases	Non-viral delivery systems for preclinical studies examining therapies for restoring female fertility or treating reproductive diseases
Ovaries (OV)	On either side of the uterus	Produce eggs, oestrogen, progesterone	• ZIKV, HSV-1 and VACV detected in follicles and adjacent ovarian stromal tissues in mice (83-85) • HBV infection of ovarian immune cells in the human ovary (86)	No viral delivery systems reported.	• Gold NPs accumulate in murine ovaries and uterus (81) • Lipidots (cholesterol-loaded LNPs) biodistribute in the corpus luteum bodies of the ovaries, not in other parts of the gland (87)
Upper reproductive tract	Upper cervical region including the ovaries, fallopian tubes, and uterus	Fertilization, embryo development, implantation	No evidence of viral entry reported.	No viral delivery systems reported.	No non-viral delivery approaches reported.
Cervical transforma-tion zone (TZ)	Separation between the upper and the lower cervix	Blocks ascending pathogens	• HPV infects TZ during cervical epithelial eversion (88)	No viral delivery systems reported.	No non-viral delivery approaches reported.
Lower reproductive Tract	Vaginal and lower cervical regions	Acidic, antimicrobial, immune protection	• Papillomaviridae frequently observed within the lower reproductive tract (89)	No viral delivery systems reported.	No non-viral delivery approaches reported.
Female reproductive tract (FRT) epithelial cells		Barrier of epithelial cells	No evidence of viral entry reported.	No viral delivery systems reported.	No non-viral delivery approaches reported.
Cervical mucus	Within the cervix	Prevents ascending pathogens	No evidence of viral entry reported.	No viral delivery systems reported.	• PBA-LNPs enable selective mRNA delivery and genome editing of cervical cancer cells overexpressing sialic acid (90)
Blood-Follicle Barrier (BFB)	Surrounding the ovarian follicles (oocytes)	Protects oocytes; filters by size and charge	No evidence of viral entry reported.	• SeV-mediated RNA delivery bypassed defective BFB and restored oogenesis in infertile mice without germline integration (76)	No non-viral delivery approaches reported.

Structure/ Region	Location	Function	Known viral infection mechanism	Viral delivery systems for preclinical studies examining gene therapies restoring female fertility or treating reproductive diseases	Non-viral delivery systems for preclinical studies examining therapies for restoring female fertility or treating reproductive diseases
				• AdV vectors penetrate the BFB upon ovary injection by an unclear mechanism (91) • AAV9, injected into the mouse ovarian stroma, penetrates the BFB and infects granulosa cells, likely via transcytosis (77)	
Theca cells	At the outside of granulosa cells	Androgen production	• ZIKV RNA detected in granulosa and theca cells of immunocompromised ZIKV infected mice (92)	No viral delivery systems reported.	• Upon intravenous administration in mice, 80 nm LNPs penetrate ovarian cells until the basal membrane and the thecal layer, suggesting their inability to cross the BFB (81) • mRNA-LNPs mainly observed in theca cells and connective tissue upon IV injection, no mRNA detection in oocytes of rats and cynomolgus monkey (93)
Basal lamina	Separates the follicular cells from the surrounding stroma in the ovary	Selective barrier for nutrients and signals	No evidence of viral entry reported.	No viral delivery systems reported.	No non-viral delivery approaches reported.
Granulosa cells	Surrounds the oocytes within the ovarian follicle	Supports oocyte maturation and hormone production	• HBV antigens found in human ova, granular and interstitial cells (86)	No viral delivery systems reported.	No non-viral delivery approaches reported.
Cumulus cells	Surrounding the oocyte in the ovary	Protects oocyte	No evidence of viral entry reported.	No viral delivery systems reported.	No non-viral delivery approaches reported.
Zona Pellucida (ZP)	Immediately surrounding the oocytes	Mediates sperm binding and blocks polyspermy	• Viruses infect ZP-containing oocytes less than ZP-free ones, suggesting a role for ZP as a physical barrier (94)	No viral delivery systems reported.	No non-viral delivery approaches reported.
Oocytes	Within the ovaries	Female germ cell for reproduction	• HBV DNA and RNA detected in human oocytes without cytopathic effect (95, 96)	No viral delivery systems reported.	No non-viral delivery approaches reported.

Abbreviations : AAV, adeno-associated virus; AdV, Adenovirus; BFB, Blood-Follicle Barrier; HBV, Hepatitis B virus; HIV, Human immunodeficiency virus; HPV, Human Papillomavirus; HSV-1, Herpes simplex virus type 1; IV, Intravenous; LNP, Lipid nanoparticles; mRNA, messenger ribonucleic acid; PBA-LNP, Phenylboronic acid (PBA)-derived LNP; SeV, Sendai virus; TZ, Cervical transformation zone; VACV, Vaccinia virus; ZIKV, Zika virus; ZP, Zona Pellucida ; Note: “No evidence of viral entry reported” or “No (non)-viral delivery systems reported” means that, to date, no published or publicly available data describing viral or non-viral delivery approaches targeting this structure or cell type in preclinical models have been identified.

Key messages

- Depending on the mode/route of administration, viral and non-viral delivery system encounter **various barriers before reaching their target tissues/cells.** Upon systemic administration, delivery systems face the binding by plasma proteins, the clearance by circulating phagocytic cells, and the clearance by several organs like the liver, the spleen and the kidneys. In the cell, efficient delivery of the payload into the cytosol may also be hampered by endosomal entrapment, a concern for non-viral delivery systems in particular.
- Delivery systems that are purposely **designed to target** specific tissues, together with the chosen **mode of administration**, can further reduce the likelihood that delivery systems inadvertently reach germline tissues or cells.
- The **male and the female reproductive system** present complex interactions of epithelial cell tight junctions and physiological and immunological components that limit effective exposure of gametes and oocytes.

4 Current Understanding, Challenges, Risks, and Future Considerations Related to Inadvertent Germline Modification

4.1 Research projects

Surveying current research efforts may aid in the identification of gaps that remain in safety assessment. A systematic review of publicly available research databases was therefore conducted, focusing on projects that address key challenges in biodistribution, delivery efficiency, and potential germline exposure. The analysis covered European, international, and US-funded initiatives, providing a broad picture of ongoing innovation. **Table 5** lists the research projects that were identified for this study.

Six projects were identified from the CORDIS database, the European Commission's primary information service for EU-funded research and innovation projects. These projects focus on overcoming biological barriers through nanoparticle-based delivery systems as a means of enhancing precision of gene therapies. This corroborates a trend toward the development of non-viral vectors to reduce immunogenicity and toxicity associated with conventional viral systems. Emphasis is made on the need for advanced biodistribution models integrating *in silico* simulations, microfluidics, and *in vivo* validation. Novel technologies for real-time, high-resolution thermal and spatial tracking of therapeutic agents *in vivo* are being explored.

A targeted search in the ERIS database, which provides structured information on projects funded by European Research Infrastructures and international collaborations, identified six projects underscoring a shift toward precise, safe, and tissue-specific *in vivo* gene therapies using AAV and non-viral delivery systems. A growing trend involves enhancing genome editing through Cas-based systems, transposases, or base-editors that reduce double-strand breaks and off-target genomic effects, while keeping an emphasis on overcoming AAV payload limitations and achieving efficient delivery to hard-to-transduce tissues. Along with remaining knowledge gaps in biodistribution assessment long-term expression in replicating tissues, and immune response management, projects show a push toward humanized models and non-human primates to improve preclinical translatability. Biodistribution studies are increasingly aim for single-cell resolution and tissue-specific profiling using advanced imaging and -omics tools.

A targeted search in the NIH RePORTER database, the US National Institutes of Health's open-access tool for tracking federally funded biomedical research, also identified six NIH-funded projects focused on advancing somatic cell genome editing technologies for the treatment of human diseases. These initiatives focus on evaluating the safety, efficacy, and biological effects of genome editing in preclinical models, ensuring rigorous testing before translation to clinical applications. Broadly, these projects address the need for high-quality animal models that closely mimic human physiology, by optimizing delivery methods and assessing immune responses. Standardized protocols, testing services, and collaborative resources are meant to provide a robust framework for translational research.

Overall, the field is progressing toward personalized and adaptive delivery systems, with growing interest in the potential of non-viral delivery systems. Within the scope of this study, no research project was identified that specifically investigated biodistribution to gonadal tissues. Nonetheless, there is a recognized need for standardized and scalable methods to evaluate

biodistribution and pharmacokinetics of novel gene therapy delivery systems in complex biological environments.

Table 5. Selected research projects

Project title	Lead Organisation	Reference
BIOmaterial RIsk MAnagement (BIORIMA)	University Ca' Foscari of Venice, Italy	CORDIS: 760928 https://cordis.europa.eu/project/id/760928
Evolvable platform for programmable nanoparticle-based cancer therapies (EVO-NANO)	University of Bristol, United Kingdom	CORDIS: 800983 https://cordis.europa.eu/project/id/800983
Supramolecular Polyamine Gene Vectors for Cancer Therapy (SUPRO-GEN)	Centro de Investigación Cooperativa en Biomateriales CIC biomaGUNE, Spain	CORDIS: 101008072 https://cordis.europa.eu/project/id/101008072
Accelerating Research & Development for Advanced Therapies (ARDAT)	University of Sheffield, United Kingdom	CORDIS: 945473 https://cordis.europa.eu/project/id/945473
In-vivo Gene Editing by NanotransducErs (I-GENE)	University of Pisa, Italy	CORDIS: 862714 https://cordis.europa.eu/project/id/862714
New Prime Editing and non-viral delivery strategies for Gene Therapy (EdiGenT)	Sorbonne Université, France	CORDIS: 101070903 https://cordis.europa.eu/project/id/101070903
EXPanding AAV gene therapy by EDITing (EXPEDITE)	Fondazione Telethon ETS, Italy	ERIS: ERC-101097155 https://cordis.europa.eu/project/id/101097155
EYEGET (EYE GEne Therapy)	Fondazione Telethon ETS, Italy	ERIS: ERC-694323 https://cordis.europa.eu/project/id/694323
Development of Innovative Therapeutic Strategies for betahemoglobinopathies (DITSB)	Institut national de la santé et de la recherche médicale, France	ERIS: ERC- 865797 https://cordis.europa.eu/project/id/865797
Overcoming the epigenetic and therapeutic barrier of EVI1-overexpressing cancers	Deutsches Krebsforschungszentrum Heidelberg, Germany	ERIS: ERC-677209 https://cordis.europa.eu/project/id/677209
From iPSC-Macrophage Biology Towards Regenerative Therapies Targeting Respiratory Infections	Hannover Medical School, Germany	ERIS: ERC- 852178 https://cordis.europa.eu/project/id/852178
Gene therapy of inherited and acquired hearing loss (IHEAR)	Hannover Medical School, Germany	ERIS: ERC- 819531 https://cordis.europa.eu/project/id/819531
Translational Nonhuman Primate Regenerative Medicine and Gene Therapy/ Genome Editing Resource Program	University of California at Davis, United States	**NIH RePORTER:** 5R24OD034056-03 https://reporter.nih.gov/search/Tx-fg2J8Ek2WoLwfoawHdw/project-details/10983336
Nonhuman Primate Testing Center for Evaluation of Somatic Cell Genome Editing Tools / Genome Editing testing Core	University of California at Davis, United States	**NIH RePORTER:** 5U42OD027094-05 7497 https://reporter.nih.gov/search/Tx-fg2J8Ek2WoLwfoawHdw/project-details/10599929
BCM/RICE Genome Editing Testing Center / Genome Editing and Biological Effects Testing Section	Baylor college of medicine, United States	**NIH RePORTER:** 5U42OD035581-02 8379 https://reporter.nih.gov/search/xAVkp9RxZky0ZewmguF-QQ/project-details/10917291
Center for Somatic Cell Genome Editing in Nonhuman Primates /Genome Editing and Biological effects Testing Component)	University of California at Davis, United States	**NIH RePORTER:** 5U42OD035737-02 8433 https://reporter.nih.gov/search/Tx-fg2J8Ek2WoLwfoawHdw/project-details/10911367
Swine Somatic Cell Gene Editing Testing Center / Genome Editing and Biological Effects Testing: Somatic Cell Gene Editing Testing	University of Missouri-Columbia, United States	**NIH RePORTER:** 5U42OD035738-02 8401 https://reporter.nih.gov/search/Tx-fg2J8Ek2WoLwfoawHdw/project-details/10928245
MU Rodent Testing Center for Somatic Cell Genome Editing	University of Missouri-Columbia, United States	**NIH RePORTER:** 5U42OD035739-02 https://reporter.nih.gov/search/lHLf7FH4t0-LZs8q-frnZA/project-details/11169746

4.2 Literature

The majority (~78%) of the 440 identified studies from our literature survey examined viral delivery systems, particularly AAV and lentiviral vectors. A smaller subset addressed non-viral platforms such as polymer-based and lipid-based nanoparticles. Most studies involved *in vivo* experiments, while others included *in vitro* or *ex vivo* methodologies. From the AI-assisted and customized data extraction (see Section 8.1.5), 30 publications were retained on the basis of a reported examination of biodistribution to gonads. From these, 18 studies examined exclusively viral vectors, 3 studies examined both viral and non-viral delivery systems and 10 studies examined only non-viral delivery systems. Twenty-four included *in vivo* research, while 11 reported *in vitro* or *ex vivo* components. Only a few studies discussed clinical data or late-stage translational implications.

Within these 30 papers, AAV vectors were the most frequently represented (5 studies, 16.1%), followed by polymeric nanoparticles (4 studies, 12.9%), adenoviral vectors (3 studies, 9.7%), and herpesvirus vectors (3 studies, 9.7%). Smaller proportions were identified for lipid-based nanoparticles (2 studies, 6.5%), liposomes (1 study, 3.2%), lentiviral vectors (1 study, 3.2%), and non-viral transposon systems (1 study, 3.2%). The remaining 14 studies (45.2%) employed other delivery systems not captured by these categories (e.g., naked DNA, ORFV-based viral systems, or mixed viral and non-viral approaches).

Of the 30 studies, 27 included some form of biodistribution assessment. Methods used to evaluate tissue distribution varied, including:

- Bioluminescence imaging (BLI)
- Quantitative PCR (qPCR)
- Reverse transcription PCR (RT-PCR)
- Reporter gene tracking (e.g., GFP, NanoLuc)
- Nucleic acid-based detection (delivery system-specific DNA or RNA quantification)

These methods were used to assess systemic distribution and organ-specific accumulation, including unintended uptake in reproductive tissues.

Although the retained studies explicitly mentioned or examined potential exposure to gonadal or germline tissues, the depth of analysis varied considerably across them:

- 12 studies included direct analysis of gonadal tissue or reproductive cells.
- 9 reported no detectable presence of delivery system DNA or gene-editing components in gonads.
- Several others called for more research on potential germline integration risks, particularly in the context of long-term expression delivery systems such as AAV.

While none of the studies reported confirmed germline modification, the extended persistence of non-replicative viral vectors - particularly episomal AAV-remains a potential concern.

4.3 Gene Therapy Study Summaries by Delivery System Type

4.3.1 AAV vectors

Several studies highlight the tissue-specific tropism and overall safety of AAV-based vectors, particularly in relation to gonadal exposure. In a GLP-compliant preclinical program using an

AAV8 vector encoding human Ugene therapy 1A1 for Crigler-Najjar syndrome, low but persistent vector genome copies were detected in the gonads up to 6 months post-injection. However, no integration was found in oocytes, and transient germline shedding in male rabbits resolved within 30 days (97). A related dual AAV8 study delivering the MYO7A gene to non-human primates via subretinal injection showed vector DNA restricted to ocular tissues and visual pathways, with no detectable presence in the testes, ovaries, or epididymis (98).

Regulatory and analytical guidance documents reinforce that recombinant AAV vectors persist episomally and exhibit low integration rates. Moreover, AAV vectors containing gene-editing components present an elevated risk of integration into the host genome (3, 99).These properties necessitate careful biodistribution studies, often using qPCR, with reproductive organs routinely included as non-target safety endpoints. Although gonadal presence is sometimes detected, it is typically low and transient, prompting follow-up only when persistence or expression is evident (100). As a precaution, current recommendations advise against administering AAV vectors containing gene-editing components directly into gonadal tissues or organs in clinical trial participants, in order to minimize the potential for unintended germline modification (101).

In studies utilizing systemic administration, AAV DNA was frequently detected in the liver, spleen, and occasionally in the gonads, especially at high vector doses (102). For instance, AAV2 vectors administered intramuscularly or intravenously showed presence in reproductive tissues up to 90 days in rabbits and mice. However, in primates, vector DNA was rarely detected in gonadal tissue, and when present, it was transient and not associated with germline integration. For example, rAAV5 administered to non-human primates resulted in transient detection of vector genomes in semen (day 8 only) but not in gonadal tissues at later time points (103).

AAV9P31, a variant capable of crossing the blood-brain barrier, showed effective targeting of the central nervous system (CNS) in mice, with minimal detection in reproductive tissues such as the uterus and prostate, and no signal observed in the testes (104). In this preclinical safety and biodistribution study, AAV9P31-delivered CRISPR components were evaluated in rhesus macaques following intravenous administration. Biodistribution was evaluated using qPCR targeting the vector genome, with a limit of detection (LOD) of 10 copies/µg of genomic DNA. The study examined over 25 tissues, including key reproductive organs such as the testes, prostate, and uterus. No vector DNA was detected in the testes at any dose level, while only trace amounts - just above the LOD - were found in the prostate and uterus. These findings suggest minimal biodistribution to gonadal tissues and support the vector's CNS-targeting profile with limited off-target tissue exposure.

Biodistribution of AAV vectors delivered locally, such as via subretinal or intrathecal injection, was generally restricted to the injection site and nearby tissues. For instance, subretinal delivery of dual AAV8 vectors in cynomolgus monkeys resulted in negligible systemic spread and no vector presence in the gonads (98). Biodistribution was assessed using qPCR targeting the AAV genome in over 50 tissues, with a LOD of 10 copies/µg of genomic DNA. Vector DNA was predominantly detected in ocular tissues and components of the visual pathway, with no detectable vector genome copies in the testes, ovaries, or epididymis, confirming the localized nature of transgene distribution following subretinal delivery.

4.3.2 Adenoviral Vectors

Adenoviral vectors are commonly used in gene therapy due to their high transduction efficiency, but although replication-deficient, adenoviral vectors have broader biodistribution patterns and higher immunogenicity compared to AAV. Following systemic delivery, vectors distribute widely

across organs, with several studies reporting the presence of vector genomes in liver, spleen, and sometimes in gonads depending on dose and administration route.

The RIVM report compiles multiple studies in which adenovirus type 5 vectors were administered intravenously or intramuscularly to animals (105). It notes that low levels of vector DNA were detected in the testes of rabbits and mice following intramuscular administration; however, germline transmission was not confirmed. While occasional gonadal exposure was observed, vector presence was generally transient and did not result in heritable genetic changes.

One referenced study reported vector detection in the epididymis, which may represent a potential pathway of exposure, though not integration (106). In this study, adenoviral vectors encoding a *lacZ* reporter were delivered locally to the arterial wall of New Zealand White rabbits via intravascular and periadventitial routes, mimicking clinical angioplasty and vascular surgery procedures. Transgene biodistribution - including to gonadal tissues - was assessed using X-Gal histochemical staining and nested real-time polymerase chain reaction (RT-PCR), with a detection threshold as low as 0.01% transfection efficiency. LacZ expression was confirmed in the testis and epididymis after intravascular delivery, and in the testis alone after periadventitial administration, indicating vector leakage and systemic dissemination despite localized delivery. Biodistribution of adenoviruses appears to be influenced by chimeric antigen receptor (CAR) presence, with notable species differences. In humans, binding to erythrocytes may limit biodistribution compared to animal models (107-110).

4.3.3 Lentiviral Vectors

Lentiviral vectors are often chosen for stable gene transfer due to their high integration capability. In a study involving lentiviral vectors targeting ovarian tumours, vector accumulation was observed in the liver, spleen, and tumour tissues, with no evidence of integration or expression in the ovaries. (111). The study used Balb/c nude mice implanted with SKOV-3m human ovarian carcinoma xenografts. A streptavidin-displaying lentivirus was conjugated with biotinylated, ^{111}In-labeled Cetuximab and administered intravenously. Biodistribution was tracked using SPECT/CT imaging over six days. Postmortem analyses included gamma counting, quantitative PCR for vector integration, and ELISA for GFP transgene expression. Although vector DNA was detectable in ovarian tissue by qPCR, GFP expression remained below the ELISA detection limit (~30 pg/mL), indicating no active transgene expression.

4.3.4 Herpesvirus Vectors

Herpesvirus-based vectors, particularly those derived from herpes simplex virus type 1 (HSV-1), have been investigated for oncolytic and gene therapy applications due to their large genetic payload capacity. In a preclinical safety study of G207, a replication-competent HSV-1 vector, the virus was delivered intraprostatically to male mice and cynomolgus monkeys (112). Biodistribution analyses were performed using qPCR targeting the HSV DNA polymerase gene to detect viral genomes in tissues. The assay had a LOD of approximately 10 copies/µg of DNA. No viral DNA was detected in the testes of either species, and histopathological examination showed no gonadal abnormalities. These findings support the authors' conclusion that, under the conditions tested, localized HSV delivery was associated with minimal systemic spread and a low reproductive risk.

4.3.5 Lipid-Based Nanoparticles and Liposomes

Lipid-based delivery systems such as LNPs and liposomes are increasingly explored for non-viral gene therapy applications due to their modular design and lower immunogenicity. In a 2003 preclinical biodistribution study, ^{99m}Tc-radiolabeled liposomes were used to track systemic

delivery in rats. The study aimed to evaluate the organ-level distribution and clearance of the liposomal formulation. Radiolabel imaging showed preferential uptake by liver and spleen, with no significant accumulation in gonadal tissues (113).

An *in vitro* toxicology study on lipid-core nanocapsules found internalization by bovine sperm and dose-dependent toxicity, emphasizing potential germline exposure risks (114). In this study using ICR mice, intravenous injection of plasmid DNA complexed with branched PEI (2 kDa at N/P 80:1 and 25 kDa at N/P 10:1) led to detectable mIL-2 mRNA expression in ovarian tissue, peaking at 4 days and persisting up to 8 days post-injection. No expression was observed with naked DNA, and while testes were analysed, specific expression data for them were not reported.

A recent study using zwitterionic LNPs for CRISPR-Cas9 delivery to cancer models showed tumour- and liver-specific distribution (115). In this *in vivo* mouse study, which aimed to evaluate targeted gene editing efficacy, BALB/c mice bearing subcutaneous MC38 tumours were treated with systemically administered LNPs encapsulating Cas9 mRNA and sgRNA. Biodistribution was assessed using IVIS imaging of Cy5-labeled mRNA, and gene editing was evaluated by amplicon sequencing in tumour and liver tissues. Reproductive tissues, including testes and ovaries, were not directly analysed, and thus no specific data on gonadal biodistribution were reported. However, the imaging and sequencing data indicated minimal off-target distribution outside the liver and tumour, suggesting a favourable targeting profile. No quantifiable signals or editing events in gonadal tissues were described, though a formal LOD was not defined in the study.

4.3.6 Polymeric Nanoparticles

Polymeric carriers, such as dendrimers and PEIs, offer customizable architectures for nucleic acid delivery. In a biodistribution and toxicity study, PEGylated PAMAM dendrimers labelled with iodine-125 were intravenously administered to male BALB/c mice (6–8 weeks old)(116). Organ-specific accumulation, including in the gonadal tissues, was quantified using gamma (γ) counting of excised organs at multiple time points post-injection (1, 4, 8, 24, and 48 hours). Each organ, including the testes, was weighed and its radioactivity measured using a γ-counter, with results expressed as percentage of injected dose per gram of tissue (%ID/g). The limit of detection for radioactivity was approximately 0.01% ID/g. Low-level radioactivity was detected in the testes, but histopathological analysis revealed no observable tissue abnormalities.

In another study, systemic delivery of interleukin-2 plasmid DNA complexed with branched PEI was tested in mice (114). Researchers sought to examine gene expression patterns and tissue distribution, including in reproductive organs. Female outbred mice (5–6 weeks old) were administered 50 µg of plasmid DNA intravenously via tail vein injection. Gene expression levels were assessed using quantitative and competitive RT-PCR, and biodistribution was measured by qPCR following tissue DNA extraction and purification. Transient mRNA expression in the ovaries was detected, peaking at day 4 and persisting up to 8 days post-injection in mice treated with either branched PEI25K at N/P 10:1 or branched PEI2K at N/P 80:1. The analytical approach was sensitive enough to detect plasmid levels at sub-nanogram concentrations per tissue sample. Although no histological abnormalities were reported upon repeated administration, these findings demonstrate that gonadal tissues may be briefly transfected depending on the PEI formulation and dosing parameters.

4.3.7 Non-Viral Transposon Systems

The Sleeping Beauty (SB) transposon system offers a non-viral platform for integrating genetic material into host genomes. In a preclinical evaluation of CD19-directed CAR cytokine-induced killer (CIK) cells, researchers used plasmid DNA encoding the SB transposase and CD19 CAR transgene, delivered via electroporation into human peripheral blood mononuclear cells - without

the use of viral vectors or chemical carriers (117). The study aimed to evaluate tumour-targeting efficacy and systemic safety. Male and female NSG (NOD/SCID/IL2Rγnull) mice were used as the animal model, with human CD19+ leukaemia cells introduced to establish systemic disease. Although this example involves *ex vivo*–modified cells and thus falls outside the primary scope of this report, it is included here as an illustration of a distinct non-viral gene delivery system with potential future relevance for *in vivo* applications.

Biodistribution was assessed using qPCR to detect transgene sequences in various tissues. The LOD was approximately 3–10 copies/ 100 ng of genomic DNA. Modified cells persisted primarily in hematopoietic organs such as spleen and bone marrow. Although no signal was detected in reproductive tissues, the study did not specifically assess gonadal biodistribution, limiting the ability to draw definitive conclusions regarding reproductive safety.

4.4 Position papers and reviews

Several position papers and scientific reviews stress the importance of including gonadal tissues in biodistribution and safety assessments for gene therapy products. A review published in *The AAPS Journal* outlines current bioanalytical strategies for evaluating AAV-based therapies, offering guidance on monitoring delivery system DNA, RNA, and protein. It highlights that although AAV viral vectors do not integrate into the genome, they can persist episomally in non-dividing cells, including those in reproductive organs. This potential for long-term exposure, even without integration, supports the need to include reproductive tissue analysis in preclinical evaluations. (100).

A related industry white paper from 2007 (118) consolidates general principles for the preclinical safety evaluation of gene therapy delivery systems, especially focusing on detection methodologies and regulatory expectations. The document recommends that PCR-based assays and histological evaluation be applied systematically to gonads and other non-target tissues, particularly when systemic vector delivery is involved. This guidance reflects longstanding regulatory concerns over germline transmission risks, even with replication-deficient and episomally persistent vectors.

These reviews and regulatory summaries provide consistent recommendations for integrating reproductive tissue analysis, long-term vector tracking, and sensitive detection methods (e.g., qPCR, *in situ* hybridization) into safety study design. These measures ensure that both systemic biodistribution and potential germline exposure risks are adequately assessed prior to clinical application.

4.5 Expert Inquiry

To collect firsthand insights and unpublished experiences, a targeted group of experts was consulted during this study. Although the number of respondents (7) was limited, their input provided valuable, context-rich perspectives spanning all key stakeholder groups involved in emerging gene therapy applications, including regulatory authorities, professional associations, academic researchers, and clinical investigators. A qualitative analysis of the responses was conducted to identify recurring themes, areas of consensus and divergence, and perceived gaps within the current regulatory and scientific framework.

Key insights and observations derived from the inquiry are summarized below:

- A representative from a national medicines agency cited multiple international guidance documents that address the potential risk of unintended germline modifications linked to gene therapy products. The respondent highlighted the critical **importance of biodistribution**

studies in animal models, noting that the outcomes of these studies play a central role in risk assessment.

- Two representatives from separate national medicines agency expressed that, based on current scientific understanding and available data, it remains challenging to draw well-informed conclusions regarding the risk of unintended germline modifications when non-viral delivery systems are used in gene therapy. They emphasized that the mechanisms and biodistribution patterns of non-viral vectors are not yet sufficiently characterized to support a robust risk assessment, highlighting the **need for further research and regulatory guidance** in this area.
- In relation to risk mitigation strategies, one respondent noted that participants enrolled in clinical trials involving novel gene therapies delivered via non-viral methods are required to adhere to **strict reproductive precautions**. Specifically, they must use effective contraception for a predetermined duration and are prohibited from donating sperm during this period. These measures aim to minimize the potential risk of unintended germline transmission. Furthermore, the respondent recommended the incorporation of **long-term follow-up** protocols for trial participants who are of child-bearing potential, to monitor any delayed or unforeseen effects that may arise post-treatment and to strengthen the overall safety framework of such trials.
- A respondent acknowledged the persistent challenge of extrapolating data from animal models to human physiological processes, particularly in the context of reproductive biology and toxicology. In light of this limitation, the respondent highlighted recent advancements in ***in vitro*** **culture technologies**, such as organoids and organ-on-chip systems, which are emerging as promising tools for research. These innovative platforms offer more physiologically relevant models of human tissues and organs and may serve as valuable alternatives or complementary approaches to traditional animal studies. The respondent emphasized their potential utility in investigating human reproductive diseases and assessing reproductive toxicity, suggesting that such systems could enhance the predictive power and ethical standards of preclinical research.
- A principal investigator currently overseeing a clinical trial involving a novel non-viral gene therapy explained that **confidentiality agreements** in place prevent the disclosure of unpublished data. As a result, certain information - particularly regarding biodistribution patterns and the potential for unintended germline modification - may remain inaccessible to the public for the foreseeable future. This limitation underscores the broader challenge of ensuring transparency in early-stage research while balancing the need to protect proprietary data and comply with ethical and regulatory obligations.

4.6 Current Clinical Developments – *in vivo* gene editing

Among gene editing applications currently progressing through clinical development, two primary delivery systems have emerged: AAV vectors and LNPs, the latter representing the most advanced non-viral delivery system to date. Non-viral platforms, particularly LNPs, are demonstrating considerable momentum in clinical trials, with a growing number of programs targeting a range of genetic disorders. Early clinical data consistently indicate promising safety and efficacy profiles within targeted tissues. However, despite these encouraging findings, comprehensive data on biodistribution and the potential for unintended germline modification remain limited. The following section provides a synthesis of the available evidence from clinical-stage programs, with a focus on tissue specificity and the potential for germline exposure. The information presented in the following paragraphs has been sourced primarily from company press releases and specialized pharmaceutical news outlets. These sources reflect corporate or

industry communications and, unless otherwise supported by a cited scientific reference, do not constitute peer-reviewed reporting of the underlying data.

4.6.1 *In vivo* gene editing mediated by AAV

Some CRISPR-Cas gene editing therapies using AAV vectors as delivery system have entered the clinical phases.

EDIT-101 a CRISPR-Cas9-based gene-editing therapy for Leber Congenital Amaurosis type 10 (LCA10) by **Editas Medicine**, utilizes an AAV serotype 5 (AAV5) vector to target photoreceptor cells in the retina,

- No viral presence in semen has been shown despite shedding into blood, nasal mucosa and tears.
- While non-clinical biodistribution studies have not yet been published, shedding analysis from a phase I/II clinical trial has detected viral genomes in tears, nasal mucosa, and blood but not in semen.

HuidaGene Therapeutics is actively advancing a diverse portfolio of clinical programs leveraging its proprietary AI-powered CRISPR platform, HG-PRECISE®, which includes both DNA and RNA editing technologies.

- HG302 is a CRISPR-Cas12-based DNA-editing therapy for Duchenne muscular dystrophy, delivered by an AAV vector. It has been granted orphan drug designation and rare paediatric disease designation by the FDA. Neither biodistribution studies nor data specifically investigating the potential risk of germline editing were available.
- HG202 a CRISPR-Cas13-based RNA-editing therapy packaging in one-single AAV vector and targeting knock-down of VEGF-A in patients with Neovascular Age-related Macular Degeneration (nAMD) enters clinical development with two phase I trials. Neither biodistribution studies nor data specifically investigating the potential risk of germline editing were available.

4.6.2 *In vivo* gene editing mediated by LNP

Intellia Therapeutics' lead candidates utilize LNPs to deliver CRISPR-Cas9 components systemically to the liver.

- NTLA-2001, targeting TTR for the treatment of hATTR, has shown significant and sustained reductions in serum TTR levels with a favourable safety profile in early clinical trials. However, biodistribution or germline safety data have not been publicly released.
- NTLA-2002 was designed to knock out the KLKB1 in liver cells for the treatment of HAE. The phase II study was placed on an FDA hold in 2023 due to concerns arising from foetal development findings in mice. Although no public data address potential germline editing risks, the company asserts preclinical studies indicate no impact on germline cells. As of January 2025, the first patients have been enrolled in the global Phase 3 clinical trial.
- NTLA-3001 targets lung disease associated with AATD. This *in vivo* CRISPR-Cas9-based gene editing therapy received regulatory authorization in July 2024 to initiate a phase I/II clinical trial but was discontinued in early 2025 due to prioritization of other sponsor programs. Biodistribution data remain unavailable.

Verve Therapeutics has produced the most detailed preclinical biodistribution and germline analyses to date.

- VERVE-101 (LNP) and VERVE-201 (GalNAc-LNP) target PCSK9 and ANGPTL3, respectively. In non-human primates, editing was primarily confined to the liver.

- VERVE-101 revealed no detectable evidence of PCSK9 gene editing within the germline. In parallel, genotyping of 436 offspring of female mice treated with a murine surrogate of VERVE-101 demonstrated an absence of heritable transmission of the PCSK9 gene edit to offspring. Biodistribution analyses with VERVE-101 indicate that the edits are confined primarily to the liver, with negligible presence in reproductive organs (119).
- VERVE-201 biodistribution analysis demonstrated low-level editing in adrenal and spleen tissues, with minimal or undetectable editing in reproductive organs.

YolTech Therapeutics' LNP-based therapies are in early-stage trials:

- YOLT-203, targeting *HAO1*, showed low but significant levels of gene editing in several non-liver tissues, including the spleen, heart, kidney, muscle, and lung in rats. Editing in the testis was not significant, and data on the ovaries were not reported.
- YOLT-201 and YOLT-204 currently lack published studies or reports addressing the risk of germline editing.

CRISPR Therapeutics is developing CTX310 and CTX320, two LNP-delivered CRISPR-Cas9 therapies for lipid disorders.

- Biodistribution studies in non-human primates for each therapy show that gene editing is highly specific to the liver, with no detectable editing above the limit of detection in most extrahepatic tissues, including the ovary and testis.
- Furthermore, analysis of sperm from sexually mature male non-human primates treated with each therapy revealed no evidence of germline editing.
- Genomic assessments in primary human liver, spleen, and adrenal cells revealed no off-target genome editing and no chromosomal translocations.

Beam Therapeutics' BEAM-301 and BEAM-302, liver-directed base editing therapies for metabolic diseases, are in early trial stages.

- Public data on biodistribution and germline editing risks are not yet available.

Precision BioSciences' PBGENE-HBV, using the proprietary ARCUS® platform and LNPs to target integrated HBV DNA in hepatocytes, is in Phase I.

- Non-human primates biodistribution studies indicate no delivery to germ cells.

Key messages

- The limited number of peer-reviewed publications addressing biodistribution to gonadal tissues - only 30 out of a selection of 440 reviewed - highlights a **significant gap in publicly available scientific literature** concerning potential germline exposure. While none of these studies reported evidence of germline editing events, the scarcity of data suggests that additional insights may be found outside traditional academic sources. In particular, grey literature, such as conference presentations, regulatory submissions, and company disclosures, has begun to offer supplementary information on biodistribution and germline safety. It is also likely that more comprehensive data exist within regulatory safety packages submitted to health authorities; however, these materials are not yet publicly accessible. This situation underscores the importance of increased transparency and data sharing to support informed risk assessment and regulatory decision-making.
- While early clinical data from gene editing therapies utilizing AAV vectors or LNP delivery systems demonstrate **encouraging safety and efficacy profiles** in targeted tissues, regulatory authorities continue to approach these technologies with caution - particularly regarding the potential for unintended germline modifications. Preclinical studies, including

those conducted by Verve Therapeutics, have provided reassuring evidence suggesting **minimal to no germline transmission** of edits. However, for many investigational candidates, comprehensive germline safety data remain either limited or unpublished.

- In viral delivery systems, biodistribution to reproductive tissues is typically assessed using **nucleic acid-based methods**. For non-viral delivery systems, such as LNPs, **imaging** techniques like fluorescence, bioluminescence, or MRI are commonly used. However, the literature rarely provides details on the **sensitivity** of these methods - such as limits of detection or quantification - and often lacks discussion on the biological **relevance** of detecting delivery system presence in gonadal tissues.
- Only a limited number of ***in vivo* animal studies** suggest that certain delivery systems may cross biological barriers in male and female reproductive organs. Even when persistent presence in gonadal tissue is observed, confirming unintended germline modification would require high-throughput DNA sequencing of gametes or oocytes. To date, **no direct evidence of such germline editing** has been reported in published *in vivo* research.
- Demonstrating the **absence of rare events** - such as unintended germline modifications - is inherently difficult, especially in preclinical studies involving multiple species, small sample sizes, and often unreported assay sensitivity. These limitations make it challenging to confidently rule out low-frequency events.

5 Governance, oversight, international guidelines and recommendation

As gene therapy and genome editing technologies continue to evolve rapidly, ensuring safety alongside innovation remains a central priority. Governance and oversight frameworks play a key role in balancing scientific progress with public trust, while international guidelines help steer development along established, practical standards. This chapter begins with a brief overview of the current governance landscape, followed by a more detailed examination of existing guidelines and recommendations for gene therapy products - focusing specifically on the assessment of unintended germline modifications. Ethical considerations, though highly relevant, fall outside the scope of this study.

5.1 Governance and oversight

With the rise of genome editing technologies, various institutions, associations, and regulatory authorities have established positions on three distinct applications:

- somatic genome editing, which affects only the treated individual;
- human germline genome editing, involving embryos or gametes in research settings without reproductive intent; and
- heritable genome editing, where edited embryos or gametes are intended for reproduction.

Importantly, both European Union legislation and international conventions prohibit intentional germline modification for reproductive purposes. This strict prohibition provides the reference point against which regulatory authorities evaluate potential risks of gene therapy and delivery platforms. Although the aim of current therapies is restricted to somatic cells, regulators explicitly require sponsors to assess whether a therapy could lead to unintentional germline modification through biodistribution to gonadal tissues or germ cells. In other words, the regulatory stance on unintentional risks must be read in light of the absolute prohibition on intentional germline editing: because deliberate modification is not permitted, the potential for unintended modification is subject to heightened scrutiny. Accordingly, pharmacological and regulatory guidance emphasizes the need for applicants to provide data on biodistribution, germline exposure, and risk mitigation strategies as part of preclinical and clinical submissions.

A comprehensive survey of 96 countries conducted in 2020 found that 11 countries permit human germline genome editing experiments, often with private or local public funds. 23 countries prohibit them, while 56 have vague policies. None of the 96 countries explicitly permits heritable genome editing, and 70 explicitly ban it (120).

Article 13 of the Oviedo Convention[24], an international binding legal instrument and ratified by 30 countries explicitly bans heritable human genome editing. Notably, the UK and Germany are non-ratifying countries, however each prohibits heritable genome editing through national legislation. In the European Union, Directive 2001/20/EC on clinical trials on medicinal products for human use, states that no gene therapy trials may be carried out which result in modifications to the

[24] https://www.coe.int/en/web/conventions/full-list?module=treaty-detail&treatynum=164 – Accessed on 19 June 2025

subject's germline genetic identity[25]. Regulation (EU) No 536/2014, repealing Directive 2001/20/EC states that it is appropriate to maintain that provision[26].

In 2018, Chinese scientist He Jiankui provoked global condemnation after revealing the birth of twin girls whose genomes had been edited using CRISPR to confer HIV resistance. The experiment, which involved heritable genome editing, was widely criticized for its lack of transparency, ethical breaches, and disregard for scientific norms. In response, China significantly strengthened its regulatory framework. The National Science and Technology Ethics Committee, through its medical ethics subcommittee, issued updated guidelines that strictly prohibit clinical research involving germline genome editing for reproductive purposes. These guidelines emphasize a cautious and responsible approach, allowing for future consideration of such research only under rigorous supervision, with clear understanding of risks and benefits, and broad societal consensus[27].

In 2021, a report of a WHO expert advisory committee issued a framework and recommendations for governance and oversight of human genome editing (121). Furthermore, global registries ensure transparency and proper oversight[28,29].

In addition, at the Third International Summit on Human Genome Editing in March 2023, the organizing committee (UK Royal Society, UK Academy of Medical Sciences, US National Academies of Sciences and Medicine and the World Academy of Sciences) concluded that while heritable human genome editing remains unacceptable at this stage, significant progress in somatic genome editing was achieved and a more open stance could be envisaged on basic research on human germline genome editing (122). The broad and open-ended wording in a recently issued guideline from the South African Department of Health clearly appears to have sparked debate as it may have been interpreted as permitting or promoting human germline genome editing (123). In contrast, at least one company asserts that it is advancing safe and ethical research on human germline genome editing with the explicit aim of preventing or eradicating inherited diseases[30].

5.2 Guidelines on how to assess unintended germline modification

Although gene therapy products are designed to target somatic cells, unintended exposure to germline cells - and the potential transmission of genetic changes to future generations - remains a concern. This risk depends on factors such as the product type, delivery route (e.g., systemic vs. local), and dosage. International guidance documents offer recommendations for evaluating this risk, with the scope of assessment varying based on the product's development stage, target population, and therapeutic approach. **Table 6** summarizes key international guidance documents, highlighting aligned and divergent recommendations to support comparison and project-relevant insights.

[25] Directive 2001/20/EC: https://eur-lex.europa.eu/eli/dir/2001/20/oj/eng - Accessed on 19 June 2025
[26] Directive 536/2014: https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX%3A32014R0536&qid=1750345566916 – Accessed on 19 June 2025
[27] https://www.most.gov.cn/kjbgz/202407/t20240708_191311.html# - Accessed on 19 June 2025
[28]https://www.who.int/groups/expert-advisory-committee-on-developing-global-standards-for-governance-and-oversight-of-human-genome-editing/registry – Accessed on 15 September 2025
[29] https://trialsearch.who.int/ - Accessed on 12 September 2025
[30] https://manhattangenomics.com/ - Accessed on 12 September 2025

Table 66. International guidance relevant for unintended germline gene editing.

Source	Scope	Recommendations relevant for unintended germline gene editing
ICH S12, EMA/CHMP/ICH/318372/2021 Guideline on non-clinical biodistribution considerations for gene therapy products [31]	Biodistribution defined as *in vivo* distribution, persistence and clearance at the site of administration and in target and non-target tissues, including biofluids, for • Products mediating their effect by the expression (transcription or translation) of transferred genetic materials, like plasmids and RNA; microorganisms (e.g., viruses, bacteria, fungi) genetically modified to express transgenes (including products that edit the host genome), and *ex vivo* GM human cells. • Products intended to alter the host cell genome *in vivo* without specific transcription or translation (i.e., delivery of a nuclease and guide RNA by non-viral methods). • oncolytic viruses (even not GM)	• Need to conduct BD assessment in the gonads for both sexes, unless the target clinical population is restricted to one sex (e.g., treatment of prostate or uterine cancer). • In general, a minimum of 5 rodents or 3 non-rodents per sex/group/time point should be evaluated. • If the gene therapy product or its genetic material does not indicate persistence by an appropriate analytical method, further evaluation may not be necessary. • Persistent persistence is defined as the continued presence of transferred or modified genetic sequences in the host after acute exposure to a gene therapy product, due either to integration of the genetic sequence into the host genome, deletion, insertion, or otherwise modified following genome editing, to latent infection with the viral vector bearing the transgene, or to the transferred genetic material in episomal form. • Persistent presence of the gene therapy product in the gonads can lead to additional studies to determine gene therapy product levels in germ cells (e.g., oocytes, sperm) or non-germline cells in the animals. These data, as well as other factors (delivery system type, replication capacity, integration potential, dose level, ROA, etc.), can inform the risk of inadvertent germline integration or germline cell genome modification. The guidance refers to the 2006 ICH Considerations document on inadvertent germline integration of gene therapy delivery systems. • Persistent gene therapy product detection in non-germline cells in gonadal tissues (e.g., leukocytes, Sertoli cells or Leydig cells) can warrant additional consideration of its potential effect on the function of the affected non-germline cells, particularly if the cell type is important to successful reproduction. • Section 4.6. addresses the assessment of samples from injection site(s), gonads, adrenal gland, brain, spinal cord (cervical, thoracic, and lumbar), liver, kidney, lung, heart, spleen, and blood. • Section 5.1. addresses assay methodologies to quantitate delivery system genome or transgene DNA/RNA (e.g. qPCR, digital PCR) or ELISA, IHC, western blot, ISH… • Non-clinical BD assessment should be completed prior to initiation of the clinical trial and the tested article in these studies should be representative of the intended clinical gene therapy product. In some situations, non-clinical BD data generated with a gene therapy product consisting of the same delivery system intended for clinical use and a different therapeutic transgene or an expression marker gene can be leveraged to support the BD profile.

[31] European Medicines Agency. International Conference Harmonisation. S12 Guideline on nonclinical biodistribution considerations for gene therapy products. EMA/CHMP/ICH/318372/2021. September 2023. https://www.ema.europa.eu/en/documents/regulatory-procedural-guideline/ich-guideline-s12-nonclinical-biodistribution-considerations-gene-therapy-products-step-5_en.pdf

Source	Scope	Recommendations relevant for unintended germline gene editing
CHMP/ICH/469991/2006. General Principles to address the risk of inadvertent germline integration of gene therapy vectors. [32] EMEA/273974/2005. Guideline on non-clinical testing for inadvertent germline transmission of gene transfer vectors.[33]	Non-clinical inadvertent germline transmission testing for gene transfer medicinal products containing or consisting of • replication-incompetent vectors • GM viruses • nucleic acids directly administered to humans Risk is primarily addressed at the biodistribution level (signal in gonads, signal in gametes, semen fractionation studies and integration analysis)	• Only germline transmission of expression/transfer vector DNA, not RNA, is presumed to pose a risk of germline modification. A decision to study potential germline transmission depends on vector type, dose, ROA and clinical purpose. Assessment of a particular gene transfer medicinal product should be approached on a case-by-case basis. • Testing methods are gender-dependent: there is no non-invasive means to monitor women for germline transmission and therefore the assessment solely relies on non-clinical data. For men, the earlier the differentiation stage at which germline transmission takes place in the spermatogenesis process (cycle of 64-74 days in man), the greater the risk for germline modification. • Regarding in utero gene therapy, it is considered that primordial germ cells in the gonads are unprotected and mitotically active (and prone for transduction) until compartmentalisation is achieved in the 7th week of gestation. • See **Figure 3** for a decision tree for study design. No germline transmission studies is required if the population intended to be treated is definitively sterile. • Regarding, GM human cells, the risk of germline transmission is considered to be low and, as animal testing of human cells may be difficult or not meaningful, non-clinical germline transmission studies of human genetically modified cells are not recommended, unless otherwise justified (for example when the vector may be mobilised through cell death, virus infection or other means). • Negative results in sperm cells of animals can be interpreted in function of vector characteristics. No further studies may be needed for non-integrating vectors not transferring their genetic material into the nucleus. However, for vectors known to introduce their genetic material into the nucleus, testing of male patients' semen during clinical trial is encouraged. *Considerations from the authors of this report:* • The guidance is focusing on BD assessment of vector DNA, it may not have considered recent developments involving non-viral vectors with mRNA encoding for gene editing components (e.g. LNP with mRNA coding for Cas9). • According to this guideline, non-clinical pharmacology and biodistribution studies typically involve at least two species and both sexes. This requirement is less stringent than those outlined in ICH S12 (2023) and FDA's long-term follow-up guidance (2020), which generally recommend a minimum of five rodents or three non-rodents per sex, group, and time point.

[32] European Medicines Agency. International Conference Harmonisation Considerations. CHMP/ICH/469991/2006. General Principles to address the risk of inadvertent germline integration of gene therapy vectors. November 2006. https://www.ema.europa.eu/en/documents/scientific-guideline/international-conference-harmonisation-technical-requirements-registration-pharmaceuticals-human-use-considerations-general-principles-address-risk-inadvertent-germline-integration-gene-th_en.pdf

[33] European Medicines Agency. Committee for medicinal products for human use. EMEA/273974/2005. Guideline on non-clinical testing for inadvertent germline transmission of gene transfer vectors. November 2006. https://www.ema.europa.eu/en/documents/scientific-guideline/guideline-non-clinical-testing-inadvertent-germline-transmission-gene-transfer-vectors_en.pdf

Source	Scope	Recommendations relevant for unintended germline gene editing
FDA. Human Gene Therapy Products incorporating human genome editing. Jan 2024[34]	• Safety and quality assessment and how to address potential risks of gene therapy products involving genome editing (GE) • Assessing specific risks associated with GE approaches including off-target editing, unintended consequences of on-target editing, and the unknown long-term effects of on- and off-target editing	• Assessment of biodistribution should be conducted to characterize the distribution, persistence, and clearance of the GE product, any expressed GE components *in vivo*, editing activity in target and non-target tissues, and the potential for inadvertent germline modification. These evaluations may be conducted independently or in conjunction with proof of concept and/or safety studies. • Emphasizes the importance of limiting the period of functional activity for the GE component to minimize potential for both off-target and unintended on-target genome editing events. • The potential for inadvertent germline modifications is covered under BD assessment (instead of under safety assessment). If GE's product BD profile includes germline tissues, an associate on- and off-target safety data package is requested. BD data are not explicitly required under GLP.
FDA. FAQ on Cellular and Gene Therapy Products. Draft guidance, 2024[35]	The FAQ spans different disciplines and aims at providing an answer on the industry's FAQ and commonly faced issues in their development of safe, effective and high-quality products	**Section V (**non-clinical studies): **Q24** on the possibility of alternative methods in place of animal studies: 'FDA is open to alternative methods that are backed by science and produce scientifically valid data, applicant proposals should be discussed as early as possible in development of product'. **Q29** on the duration of pivotal toxicology studies for a single-dose administration investigational product: ' the study duration should be informed by the biodistribution data and persistence profile. Data should allow to evaluate potential acute and long-term toxicities and sacrifice time points should allow comprehensive characterization of potential adverse effects. **Q30** on the recommendations for testing methos for vector biodistribution vectors: quantitative and sensitive assay such as qPCR is recommended to analyse vector BD and persistence. Upon positive results, transgene mRNA and /or protein expression level should also be measured. Further refers to ICH S12 and Long-Term Follow-Up (LTFU) guidance of FDA, 2020. **Section VI** (clinical studies): **Q35** on what to consider for short- and long-term safety monitoring: immediately after administration: intensive safety monitoring with frequent monitoring of vital signs, physical examinations, laboratory studies, radiologic evaluations, and other relevant studies as warranted. Consider staggered enrolment. The idea is that LTFU is needed for gene therapy products due to their genome integration potential or base editing potential. It is further referred to LTFU guidance of FDA, 2020. *Considerations from the authors of this report*: • if BD non-clinical studies show vector presence in gonads, there are no clear recommendations as to whether genome integration (site) analysis should be performed on sperm cells of male CT participants, and for how long.

[34] US Food and Drug Administration. Guidance for industry. Human Gene therapy Products Incorporating Human Genome Editing. January 2024. https://www.fda.gov/media/156894/download

[35] US Food and Drug Administration. Guidance document. Frequently Asked Questions- Developing Potential Cellular and Gene Therapy Products. November 2024. https://www.fda.gov/media/183631/download

Source	Scope	Recommendations relevant for unintended germline gene editing
EMEA/CHMP/gene therapy WP/60436/2007. Guideline on follow-up of patients administered with gene therapy medicinal products.[36]	• Active clinical follow-up to gain insight in safety, efficacy • Applies to IMP as well as MP, on gene therapy products based on viral vectors (including oncolytic vectors), non-viral vectors and plasmids, GM cells.	• Viral vectors with potential for integration or late re-activation (as indicated by non-clinical data): monitoring plan + sample testing at pre-treatment, 3, 6 and 12 months after treatment for at least 5 years, and then yearly until data indicate that there is no longer any risk to be followed. • Viral vectors without integration, latency and re-activation potential OR non-viral vectors OR plasmids: monitoring plan + sample testing at pre-treatment, 3, 6 and 12 months after treatment, and then yearly thereafter for a minimum of 5 years. • Other factors impacting risk assessment and decision about the extent/duration of the follow-up include: ○ Potential for and extent of chromosomal integration of a vector/gene; ○ Capacity of a vector / gene for latency/reactivation; ○ Capacity of a vector for inadvertent replication after complementation by viruses causing escape; from latency and reactivation and eventually leading to mobilisation; ○ Persistence of expression of the gene/vector/gene product; ○ Replication incompetence or competence of a vector; ○ Potential for recombination or re-assortment; ○ Altered expression of (a) host gene(s); ○ Biodistribution to target / non-target organ(s) / tissue(s) / cell(s); ○ Known interactions with concomitant treatments or known interactions associated with previous exposure to potent agents (chemotherapy, radiotherapy etc.). *Considerations from the authors of this report:* • The guidance primarily focuses on patient safety and product efficacy. It does not explicitly address delayed effects related to potential germline transmission. • Does not specifically consider the use of novel gene therapy products involving gene editing (contrary to LTFU guidance of FDA, 2020).
EMEA/149995/2008 rev.1. Guideline on safety and efficacy follow-up – risk management of advanced therapy medicinal products[37]	This guideline describes specific aspects of pharmacovigilance, risk management planning, safety and efficacy follow-up of authorised ATMPs, as well as some aspects of clinical follow-up of patients treated with such products.	• Because of the wide range of products covered, the novelty and high speed of development in this area, applicants are encouraged to seek scientific advice for Risk Management Planning from the EMEA. • Among the risks to be discussed in the Risk management plan of an ATMP are the risks to healthcare professionals, care givers, offspring and other close contacts with the product or its components, or with patients, presented in a summary fashion and based on the environmental risk assessment. • Examples likely to represent important safety concerns: Potential of the vector for latency and reactivation, integration of genetic material into host genome, prolonged expression of the transgene, altered expression of the host's genes, potential for germline integration. *Considerations from the authors of this report:* • No specific guidance is given on how to assess potential germline integration or germline genome modification.

[36] European Medicines Agency. Committee for medicinal products for human use. EMEA/CHMP/gene therapy WP/60436/2007. Guideline on Follow-up Patients Administered with Gene Therapy Medicinal Products. October 2009. https://www.ema.europa.eu/en/documents/scientific-guideline/guideline-follow-patients-administered-gene-therapy-medicinal-products_en.pdf

[37] European Medicines Agency. EMEA/149995/2008 rev.1. Guideline on safety and efficacy follow-up and risk management of Advanced Therapy Medicinal Products. January 2018. https://www.ema.europa.eu/en/documents/scientific-guideline/draft-guideline-safety-and-efficacy-follow-and-risk-management-advanced-therapy-medicinal-products-revision-1_en.pdf

Source	Scope	Recommendations relevant for unintended germline gene editing
FDA, Guidance for industry. Long Term Follow-up after administration of human Gene therapy products. 2020[38]	• Active clinical follow-up to gain insight in safety, efficacy • Applies on IMP as well as MP, on gene therapy products based on viral vectors (including oncolytic vectors), microbial vectors, products involving gene editing products	• See decision tree in Figure 1 of the FDA guidance. In general, follow-up is recommended for ○ 15 years for integrating vectors such as gammaretroviral and lentiviral vectors and transposon elements; ○ Up to 15 years for herpes virus vectors (or oncolytics) that are capable of establishing latency; ○ Up to 15 years for microbial vectors that are known to establish persistent infection; ○ Up to 15 years for genome editing products. (section V.G, for specific considerations: in case of systemic administration, to consider monitoring of clinical safety monitoring dur to non-target effects in non-target organs, tissue. ○ Up to 5 years for AAV vectors. • The extent/duration of the follow-up of the novel gene therapy product should take into account product-specific characteristics, the basic and translational knowledge generated in the field, and the product-specific preclinical data. *Considerations from the authors of this report:* • Includes specific considerations for gene therapy products involving gene editing components. • LTFU plan should be based on non-clinical BD data, according to iV,B, 1.c at least 5 rodents /sex/group/sacrifice time point for rodents, and between 3-5 non-rodents /sex/group/sacrifice time point (higher requirements as compared to EMA guideline (273974/2005) for non-clinical BD.
EMA/CAT/22473/2025. Guideline on the quality, non-clinical and clinical requirements for investigational advanced therapy medicinal products in clinical trials[39]	• Focus on structure and data requirements for CTA for investigational ATMPs (exploratory trials) and a perspective towards marketing authorisation application (MAA)(confirmatory trials for pivotal data). • Development, manufacturing, quality control and non-clinical aspects are addressed and to some extent clinical aspects. • Out of scope: ○ ERA aspects of ATMP ○ Extracellular vesicles/ cellular fragments originating from human origin and chemically synthetised therapeutic sequences (as not considered ATMP)	• For genome-editing clinical trials, the principles outlined in this guideline apply and specific safety concerns should be considered, mainly due to off targeting events. At this stage the experience is too limited to provide detailed guidance. • Risk-based approach: The extent of the quality, non-clinical and clinical data can be adapted having regard to the identified potential risks. At the beginning of product development, the sponsor can perform an initial risk analysis based on existing knowledge on the type of investigational product and its intended use. The extent of quality, non-clinical and clinical data to be included in the CT submission should be commensurate with the level of risk. • For assessment of germline transmission and modification, for which the extent of studies will depend on the type of investigational gene therapy MP and its distribution to the gonads, the guidance refers to EMEA/273974/2005, EMEA/CHMP/ICH/469991/2006 and also ICH S12. • Where appropriate, animal testing should be replaced by *in vitro*, *ex vivo* or *in silico* studies or a combination thereof. For example, the development and use of cell- and tissue-based models including 2D and 3D tissue-models, organoids and microfluidics should be considered, especially for evaluating the mode of action. • Full validation of the bioanalytical methods may not be needed before first clinical study. However, sufficient information on the suitability of the used method e.g. specificity and sensitivity (limit of detection) should be provided. Full validation is expected to support later phase clinical development. *Considerations from the authors of this report:* • Regarding BD and investigation of persistence and clearance this recent guidance refers to former guidances, there are no changes in the approach to be taken. Notably, replacement of animal testing is explicitly encouraged.

[38] US Food and Drug Administration. Guidance for industry. Long Term Follow-Up After Administration of Human Gene Therapy products. January 2020. https://www.fda.gov/media/113768/download

[39] European Medicines Agency. Committee for Advanced Therapies. EMA/CAT/22473/2025. Guideline on quality, non-clinical and clinical requirements for investigational advanced therapy medicinal products in clinical trials. January 2025. https://www.ema.europa.eu/en/documents/scientific-guideline/guideline-quality-non-clinical-clinical-requirements-investigational-advanced-therapy-medicinal-products-clinical-trials_en.pdf

Source	Scope	Recommendations relevant for unintended germline gene editing
EMA/CAT/80183/2014. Guideline on the quality, non-clinical and clinical aspects of gene therapy medicinal products[40]	• Guideline for MAAs of GTMPs with recombinant nucleic acids or GMOs; excludes GM cells and ERA, but principles may apply to certain gene editing and synthetic therapeutic sequences.	• **Section 5.4. (**Pharmacokinetics, PK) points out that PK should focus on the distribution, persistence, clearance and mobilization of the gene therapy MP and should address the risk of germline transmission. Pharmacokinetic studies should be where possible combined with non-clinical safety studies. For pharmacokinetic studies only validated methods such as Nucleic Acid Test assays should be used to investigate tissue distribution and persistence of the gene therapy MP. Applicants should justify the selection of assays and their specificity and sensitivity. • **Section 5.4.1.** briefly addresses to risk of germline transmission by referring to CHMP/ICH/469991/2006: assessing primarily at the biodistribution level (signal in gonads, signal in gametes, semen fractionation studies and integration analysis). • **Section 5.5.5** (DART) provides general principles, 'if the risk for germline transmission cannot be unequivocally determined according to principles as described in the guideline on non-clinical testing for inadvertent germline transmission of gene transfer vectors, then breeding studies should be performed in order to directly address whether the administered nucleic acid is being transmitted to the offspring. • 3R principles are briefly mentioned in section 5.5.5. 'In any case, flexibility needs to be applied to employ a scientifically valid testing strategy aiming clinically translatable results, in line with 3R principles. While recognising that for certain product types routine non-clinical studies on reproductive toxicity lack predictivity it is important for human risk assessment to address any limitations, uncertainties and data gaps of the testing program. *Considerations from the authors of this report:* • This guidance is not directly relevant for the current research project; however, it shows certain openness towards 3R principle.
EMEA/CHMP/gene therapy WP/125459/2006. Guideline on the non-clinical studies required before first clinical use of gene therapy medicinal products[41]	• Focus on the non-clinical studies that are required before the first use of a gene therapy MP in human subjects.	• Refers to guideline on non-clinical testing for inadvertent germline transmission of gene transfer vectors (EMEA/273974/2005). • Explicitly stated that reproductive toxicology studies are not required before first clinical use unless the biological features of the gene therapy MP and/or proposed indication and/or the characteristics of the patient population suggest a risk for reproductive organs or function.

[40] European Medicines Agency. Committee for Advanced Therapies. EMA/CAT/80183/2014. Guideline on the quality, non-clinical and clinical aspects of gene therapy medicinal products. March 2018. https://www.ema.europa.eu/en/documents/scientific-guideline/guideline-quality-non-clinical-and-clinical-aspects-gene-therapy-medicinal-products_en.pdf

[41] European Medicines Agency. Committee for medicinal products for human use. EMEA/CHMP/gene therapy WP/125459/2006. May 2008. Guideline on the non-clinical studies required before first clinical use of gene therapy medicinal products. https://www.ema.europa.eu/en/documents/scientific-guideline/guideline-non-clinical-studies-required-first-clinical-use-gene-therapy-medicinal-products_en.pdf

Source	Scope	Recommendations relevant for unintended germline gene editing
ICH S5 (R3), EMA/CHMP/ICH/544278/1998. Guideline on detection of reproductive and developmental toxicity for human pharmaceuticals[42]	• All pharmaceuticals, except for gene and cell therapy products • Focuses on ○ fertility studies ○ embryo-foetal development studies ○ pre-and postnatal development studies	• Considering risk/benefit of products intended to treat severely debilitating or life-threatening or late-life onset diseases, where the likelihood for reproduction is low, the testing strategy can include the use of qualified alternative assay (as presented in Annex 2 Fig.2 of the guideline). *Considerations from the authors of this report:* • While not applicable to gene and cell therapy products, this guidance is of interest as it shows an openness towards the use of qualified alternative assays in the EFD and MEFL testing strategy of pharmaceuticals, in an endeavour to minimize the use of animals. Annex 2, section 1,1 describes criteria for qualification of alternative assays.
EMEA/CHMP/gene therapy WP/587488/2007. Reflection paper on quality, non-clinical and clinical issues related to the development of recombinant adeno-associated viral vectors[43].	• Requirements specific for recombinant AAV that might be expected by the time of a market authorisation application	• The question of germline transmission in humans has not been fully resolved and short term DNA persistence has been observed in semen (serotype 2), therefore it is recommended that germline transmission is investigated during clinical studies and that the use of barrier contraception for a minimum of 3 months (in accordance with a normal spermatogenesis cycle) for individuals enrolled in clinical trials, is included in study protocols. *Considerations from the authors of this report:* • in a 'good practice' document specifically addressing ERA aspects of AAV, the scenario of vertical transmission is not further addressed while it is acknowledged that BD studies in animals have shown that DNA from AAV clinical vectors can be detected in gonadal DNA for a variable duration. • BD patters of AAV are dependent on the serotype of the parental virus from which the recombinant AAV is derived.
World Health Organization. (2023). Considerations in developing a regulatory framework for human cells and tissues and for advanced therapy medicinal products [44]	• Outlines fundamental principles for cell and gene therapy products to promote regulatory convergence	• Based on the risks associated with their use, a distinction is made between human cells and tissues for medical use (HCTs) (lower risk) and cell and gene therapy products (CGTP) at the other hand.

3R: principle to avoid animal experiments altogether (Replacement), to limit the number of animals (Reduction) and their suffering (Refinement) ; LTFU: Long term follow-up; GCT: Gene and cellular therapies; DART: Developmental and Reproductive Toxicology; BD: Biodistribution; NAT: Nucleic acid amplification techniques; EFD: embryo-foetal development; MEFL: Malformation or Embryo-Foetal Lethality

[42] European Medicines Agency. Committee for medicinal products for human use. ICH S5 (R3) Guideline on detection of reproductive and developmental toxicity for human pharmaceuticals. EMA/CHMP/ICH/544278/1998. February 2020. https://www.ema.europa.eu/en/documents/scientific-guideline/ich-s5-r3-guideline-detection-reproductive-and-developmental-toxicity-human-pharmaceuticals-step-5-revision-4_en.pdf

[43] European Medicines Agency. Committee for medicinal products for human use. Reflection paper on quality, non-clinical and clinical issues related to the development of recombinant adeno-associated viral vectors. EMEA/CHMP/gene therapy WP/587488/2007 Rev. 1. June 2010. https://www.ema.europa.eu/en/documents/scientific-guideline/reflection-paper-quality-non-clinical-and-clinical-issues-related-development-recombinant-adeno-associated-viral-vectors_en.pdf

[44] World Health Organization. (2023). Considerations in developing a regulatory framework for human cells and tissues and for advanced therapy medicinal products (Annex 3, TRS No. 1048). https://www.who.int/publications/m/item/considerations-in-developing-a-regulatory-framework-for-human-cells-and-tissues-and-for-advance-therapy-medicinal-products--annex-3--trs-1048

The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) is a global initiative that brings together regulatory authorities and pharmaceutical industry representatives from Europe, the US, Japan, and other regions. Its primary goal is to harmonize the technical and scientific standards used in the development, registration, and post-approval of medicines. The ICH S12 guideline contains non-binding recommendations for industry regarding non-clinical biodistribution studies for gene therapy products and defines biodistribution as the *in vivo* distribution, persistence and clearance at the administration site and in both target and non-target tissues, including biofluids such as blood and cerebrospinal fluid. Persistence is understood as the continued presence of transferred or modified genetic sequences in the host after acute exposure to a gene therapy product. This may result from integration into the host genome, genome editing events (e.g., deletions, insertions), latent infection with viral vectors carrying the transgene, or maintenance of the genetic material in episomal form.

ICH S12 considers three categories of gene therapy products:

- products mediating their effect by the expression (transcription or translation) of transferred genetic materials, like plasmids and RNA; microorganisms (e.g., viruses, bacteria, fungi) genetically modified to express transgenes (including products that edit the host genome), and *ex vivo* GM human cells;
- products intended to alter the host cell genome *in vivo* without specific transcription or translation (i.e., delivery of a nuclease and guide RNA by non-viral methods);
- oncolytic viruses (whether or not genetically modified to express a transgene).

Along with the considerations related to the integrative capacity and/or the replication competence of the product, the route of administration and the dose level, it is also emphasized that the level of evidence or the extent of data to be included in the application for a clinical trial should be proportionate to the risk. Extensive studies may not be needed if it can be reasonably assumed that the therapy remains somatic.

For example, the risk of germline transmission is considered to be low for gene therapy products consisting of genetically modified cells. As animal testing of human cells may be difficult or not meaningful, non-clinical germline transmission studies of human genetically modified cells are not recommended, unless otherwise justified (for example when the delivery system may be mobilised through cell death, virus infection or other means)[32].

A risk-based, tiered approach is clearly recommended for systematically evaluating and mitigating the potential for unintended germline exposure and modification. **Figure 3**, as originally presented in EMEA/273974/2005[33], illustrates this framework, which has been referenced in subsequent guidance documents, including ICH (2006)[32], ICH S12[31], and most recently EMA/CAT/22473/2025[39].

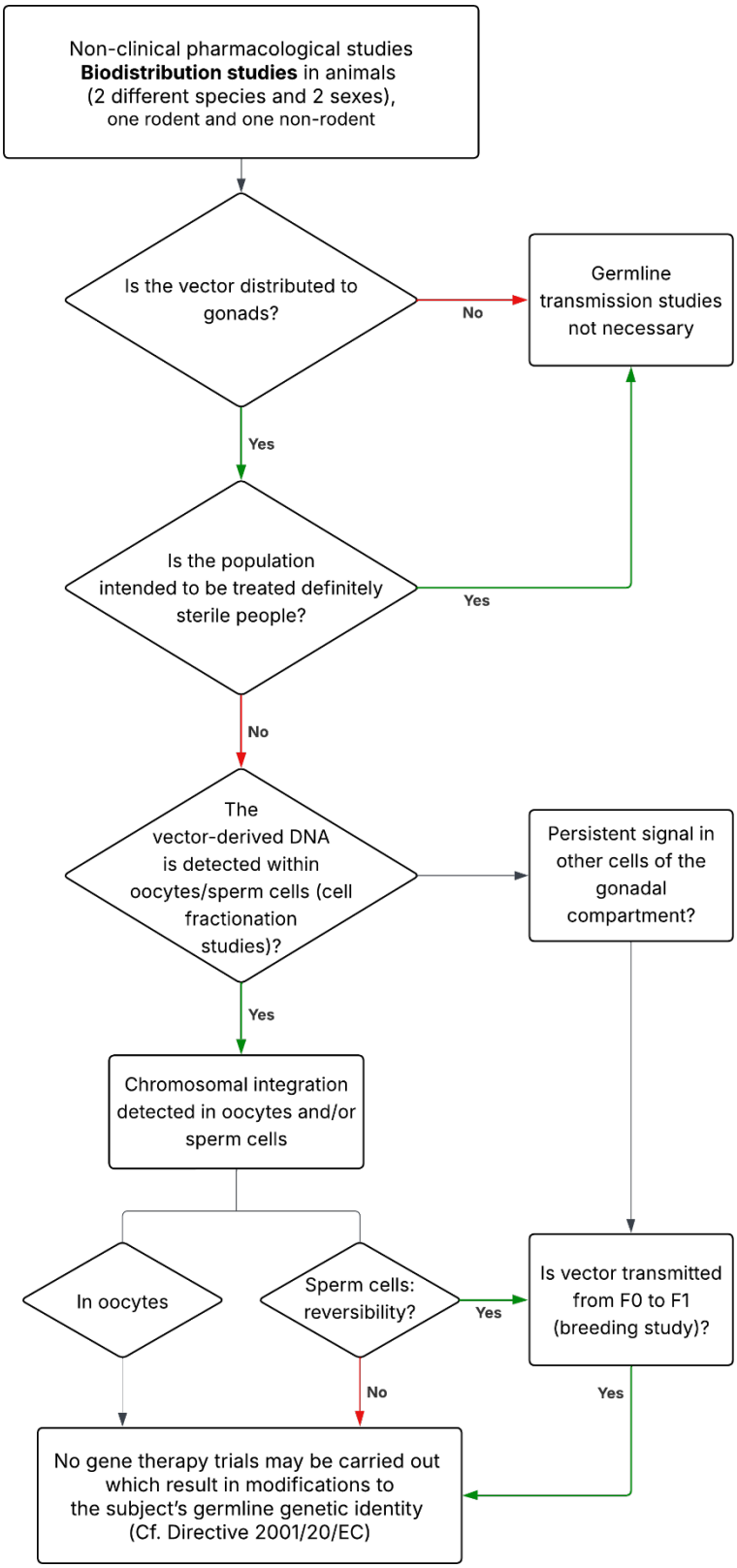

Figure 3. Flowchart on the suggested approach to studying the potential for germline transmission studies in non-clinical models. *Original chart from EMEA/273974/2005*

5.2.1 Biodistribution assessment in gonadal tissues

A starting point in biodistribution studies is the evaluation of gonadal tissues in both sexes. Prior to a first administration of a particular non-cellular gene transfer medicinal product to man, non-clinical germline transmission studies in one animal species may suffice, as indicated in EMEA/273974/2005. At the stage of marketing authorisation for non-cellular gene therapy products, EMEA/273974/2005 indicates a minimum of two species with one rodent and one non-rodent. ICH S12 recommends in general a minimum of five rodents or three non-rodents per sex, per group, per time point.

EMA/CAT/22473/2025 further advocates a risk-based approach, suggesting that the scope of quality, non-clinical, and clinical data requirements may be adjusted in accordance with the identified potential risks. At the outset of product development, sponsors are encouraged to conduct an initial risk assessment based on existing knowledge of the investigational product and its intended use. While not specified, it could be understood that existing knowledge includes available information on biodistribution of previously tested platform technologies.

Analytical methods for quantifying vector genomes or transgene DNA/RNA include qPCR, digital PCR, ELISA, IHC, western blotting, and ISH. If analytical methods indicate no persistence of the gene therapy product or its genetic material, further evaluation may not be necessary. However, persistent presence in the gonadal tissues warrants further assessment and should generally lead to additional studies such as cell fractionation studies, to determine whether the gene therapy product is present in germline cells (e.g., oocytes, sperm) or non-germline cells (e.g., leukocytes, Sertoli cells or Leydig cells) in the animals.

A positive biodistribution signal in gonadal tissues, coupled with negative results in sperm cells, should be interpreted in function of the vector's characteristics. For non-integrating vectors that do not transfer genetic material into the nucleus, further studies may not be required. However, vectors known to introduce genetic material into the nucleus necessitate germline transmission studies and may involve testing of male patients' semen during clinical trial (ICH 2006, ICH S12).

5.2.2 Germline transmission and reproductive toxicology studies

Germline transmission studies focus on detecting heritable genetic modifications, typically employing PCR assays on oocytes or sperm cells following cell fractionation. If the risk of germline transmission cannot be conclusively determined, according to principles as described in the guideline on non-clinical testing for inadvertent germline transmission of gene transfer vectors, breeding studies should be conducted to directly address whether the administered nucleic acid is being transmitted to the offspring.

Breeding studies generally fall under the scope of development and reproductive toxicology (DART) studies, which are part of standard toxicology assessments for most systemic gene therapy applications. DART studies aim to assess reproductive health and offspring well-being, employing detection methods such as histopathology, embryo viability assessments, and teratology assays. According to EMEA/CHMP/gene therapy WP/125459/2006 guidelines, reproductive toxicology studies are not required before first clinical use unless the biological features of the gene therapy product, intended indication, or patient population characteristics suggest a risk to reproductive organs or function. Also, as per EMEA/273974/2005, if biodistribution studies indicate minimal risk to reproductive organs, waivers for full reproductive

toxicity studies may be granted. However, persistent detection of the gene therapy product in non-germline gonadal cells (e.g., leukocytes, Sertoli cells or Leydig cells) can warrant additional consideration of its potential effect on the function of these cells, particularly if the cell type plays a critical role in reproduction.

From the perspective of the current project, it is noted that while biodistribution studies provide valuable input to decide on the need for germline studies and DART studies. the assessment of reproductive health is broader and has another focus as compared to the assessment of germline modification.

5.2.3 Considerations on extent of data requirements and stage of development

As per EMA/CAT/22473/2025, full validation of bioanalytical methods may not be needed before initiating the first clinical study. However, early in development, sufficient information should be provided regarding the relevance of the animal model for preclinical biodistribution studies and the suitability of the analytical methods, including specificity and sensitivity (limit of detection).

The route of administration and the dosage should reflect clinical exposure, taking into account a margin of safety. Studies may be repeated or expanded in later clinical phases, with full validation expected to support advanced clinical development.

5.2.4 Considerations on intended target population and/or risk mitigating measures

The intended clinical population for the gene therapy product influences data requirement. For example, preclinical biodistribution studies may be limited to one sex if the gene therapy products targets sex-specific conditions, such as prostate or uterine cancer (ICH S12). Additionally, if the treatment population comprises definitively sterile people, germline transmission studies may be deemed unnecessary (EMA/273974/2005).

While most regulatory guidance lacks detailed direction on risk mitigation (e.g., contraception), EMEA/CHMP/gene therapy WP/587488/2007 - specific to recombinant AAV vectors - recommends barrier contraception for at least three months post-treatment, in line with the spermatogenesis cycle. Notably, available data consistently show a lack of AAV vector detection in mature sperm or progeny.

5.2.5 Addressing novelty and rapid development in gene therapy

Existing guidelines, such as EMA/273974/2005 and the ICH guideline CHMP/ICH/469991/2006, were established before the advent of gene therapies using gene-editing technologies. These guidelines primarily consider the integration potential of the vector as a key factor in germline modification risk. While more recent guidance documents take into account emerging gene-editing therapies and their potential for unintended germline modification (FDA, 2024 and FDA, 2024 FAQ), they also recognize the relative limited experience with these types of gene therapy products, which precludes detailed guidance (e.g. EMA/CAT/22473/2025).

Given the rapid development in this field, applicants are encouraged to seek scientific advice from regulators to provide product-specific and risk proportionate guidance. Noteworthy, a recent

guideline issued by a Korean National Institute of Food and Drug safety in 2024[45] on the quality, non-clinical and clinical assessment of extravesicular vesicles exemplifies there may be an increasing need for specific guidance proper to the characteristics of the delivery system as well.

While initial guidance documents focussed on BD assessment of vector DNA, it is essential to consider to what extend this must be adapted to recent developments such as those involving non-viral vectors with mRNA encoding for gene editing components (e.g. LNP with mRNA coding for Cas9). Multiple efforts have been undertaken to reorganize and consolidate various applications, with selected examples of outlined categorisation of gene therapy applications in **Table** .

Table 7. Examples of categorisation of gene therapy applications

Guideline	Criteria	Categories
ICH 2006	Vector cellular location / integration dependency	Vectors that **enter into the cell nucleus** and that carry integration machinery
		Vectors that **enter into the cell nucleus**, without integration machinery
		Vectors that are **unable to enter** the target cell nucleus and remain cytoplasmic
ICH S12	Mode of action: expression vs. host genome modification	Products mediating their effect by the **expression** (transcription or translation) of transferred genetic materials, like plasmids and RNA; microorganisms (e.g., viruses, bacteria, fungi) genetically modified to express transgenes (including products that edit the host genome), and *ex vivo* GM human cells
		Products intended to **alter** the host cell genome *in vivo* without specific transcription or translation (i.e., delivery of a nuclease and guide RNA by non-viral methods)

In the context of unintended germline modification, it is crucial to differentiate between various application types, as technologies aimed at altering the host genome may pose higher risks - even when only a small number of vectors reach the gonadal tissues. Conversely, the risk associated with elements designed to remain cytoplasmic, such as plasmids or certain mRNA-based applications, is considered purely hypothetical under real-life conditions.

5.2.6 Commitment to the 3Rs and adoption of new approach methodologies

In alignment with Directive 2010/63/EU and the overarching objective of replacing animal research with non-animal methods wherever feasible, regulatory frameworks endorse the 3Rs principle - Replacement, Reduction, and Refinement - in the ethical use of animals for scientific purposes. Within the context of gene therapy, this mandate is further reinforced by guidance documents such as EMA/CAT/80183/2014, which outlines non-clinical requirements for advanced therapy medicinal products (ATMPs), and the more recent EMA/CAT/22473/2025, which explicitly supports the incorporation of new approach methodologies (NAMs) including organoids and microfluidic systems for evaluating biodistribution and safety. This shift reflects a broader recognition that animal models may not fully represent human physiology or predict rare but critical safety outcomes.

The integration of the 3Rs and NAMs has gained traction as a transformative strategy for biodistribution assessment, especially in ethically sensitive and biologically complex tissues such as the gonads. The limitations of 2D culture in mimicking the spatial organization and cellular

[45] Korean National Institute of Food and DRUG Safety Evaluation. Guideline on quality , non-clinical and clinical assessment of extracellular vesicles therapy products. Guidance for Industry. September 2024. file:///C:/Users/kapa525/Downloads/Guideline+on+Quality,+Non-clinical+and+Clinical+Assessment+of+Extracellular+Vesicles+Therapy+Products(Guidance+for+Industry)%20(2).pdf

behaviour of living tissues have shifted the field towards the adoption of 3D platforms. These systems enable cells to aggregate into spheroids or grow on scaffolds that stimulate the mechanical and biochemical properties of the native extracellular matrix, thereby offering a more accurate representation of *in vivo* tissue function. Together, they allow cells to adhere, stretch, and differentiate in an environment that better reflects the architecture and biomechanical cues of native tissues. However, despite their improved physiological relevance, 3D cultures are not without limitations. Habanjar *et al.* (124) highlight issues of limited reproducibility, scalability, and control over key parameters such as scaffold porosity, biocompatibility, and cell density - all of which can significantly constrain their utility in high-throughput or regulatory-compliant testing workflows.

In the specific context of gonadal tissues, these limitations become even more apparent. Although 3D cultures have enabled significant progress in modelling aspects of folliculogenesis and spermatogenesis, current systems often fail to recapitulate the full spectrum of cellular components and structural organization present *in vivo*. As Nishimura *et al.* (125) point out, most organoid models derived from human testicular or ovarian tissues are limited in their representation of mature germ cells and lack functional integration of critical elements such as vasculature or endocrine loops. The BTB, for instance, a key determinant of xenobiotic exclusion in the male gonad, has yet to be robustly reproduced *in vitro*. Although Sertoli cells are capable of forming tight junctions in 2D or partially in 3D, the assembly of a fully functional, dynamic BTB within a testicular organoid remains an unresolved challenge.

Moreover, while co-culture systems have improved our ability to examine specific cell–cell interactions - such as those between granulosa cells and oocytes - these models still fall short of fully replicating the cyclical hormonal regulation and spatial complexity of *in vivo* gonadal physiology. For example, although theca and granulosa cells have been co-cultured with oocytes in engineered 3D environments (126-128), the resulting structures rarely demonstrate complete follicular maturation or ovulatory-like events (125). Finally, although human gonadal organoids have advanced rapidly, most current models lack functional vasculature, which is essential for nutrient exchange, hormonal regulation, and immune cell trafficking.

Organ-on-a-chip (OOC) technologies offer a promising path forward. By incorporating microfluidic flow, real-time monitoring, and multi-tissue integration, OOC systems provide the dynamic and interconnected environments needed to assess biodistribution in a more physiologically relevant context (129). Several promising ovary-on-a-chip and menstrual-cycle-on-a-chip platforms that mimic hormonal cycling and follicular development under controlled flow conditions (130, 131). These systems open new possibilities for longitudinal tracking of compound kinetics and toxicity within human-derived tissues. Yet, challenges remain. Most OOC models have been validated in rodents or engineered using murine cells, and only a small subset has been adapted to fully human tissue systems. Furthermore, technical barriers related to fabrication complexity, standardization, and throughput continue to hinder their adoption for routine regulatory testing. Importantly, few of these models have undergone formal qualification under regulatory validation frameworks such as those promoted by the OECD or European Union reference Laboratory for alternatives to animal testing (EURL ECVAM).

Taken together, the integration of the 3Rs and NAMs into gonadal biodistribution assessment offers both scientific and ethical advantages but must be approached with clear awareness of their current limitations. While 3D cultures and OOC technologies bring us closer to human-relevant, mechanistically insightful platforms, their use should currently be seen as

complementary rather than replacement tools for *in vivo* studies. Strategic investment in model refinement, interlaboratory reproducibility studies, and regulatory engagement will be essential to realize their full potential as standardized approaches in ATMP development and safety evaluation.

Key messages

- The inadvertent germline exposure and possible unintended transmission of genetic changes to future generations is **considered a potential concern** depending on the nature of the product, the route of administration (e.g. systemic versus local delivery) and the dose level.
- **A tiered approach** is proposed to systematically evaluate (and mitigate) the risk of unintended germline exposure and possible germline modification in which the biodistribution to gonadal tissues in male and female animal models is considered as a first step. Further studies are warranted if there is evidence for persistent presence in gonadal tissues.
- The extent of data requirements depends on the stage of the development of the gene therapy product, the intended target population and should be **proportionate to the risk**. Standard risk mitigation measures applicable for gene therapy products should also be considered in overall risk assessment.
- There is both a **recognition of the rapid development** in gene therapy and an openness to novelty and to **new methodologies that support the 3Rs principle**.

6 Conclusion

Although *in vivo* applications of gene therapy products are specifically designed to target somatic cells, the potential for inadvertent germline modification - and the associated risk of transmitting genetic changes to future generations - remains a critical concern that warrants thorough evaluation. This issue gains particular relevance as novel non-viral delivery systems, such as lipid nanoparticles and extracellular vesicles, advance through preclinical development. These systems exhibit properties that may, at least theoretically, enable them to bypass biological barriers and reach germ cells. While the overall likelihood of such unintended germline effects remains very low, it cannot be entirely ruled out. Therefore, a comprehensive understanding of the characteristics of these emerging platforms, along with the ability to anticipate their clinical behaviour, is essential for conducting proportionate and scientifically sound risk assessments.

This study aimed to enhance understanding of the potential for inadvertent germline modification associated with novel gene therapies by reviewing data on gene delivery systems and their biodistribution to gonadal tissues, alongside evidence from animal studies. While the available information remains limited and dispersed across various sources, the authors have carefully reviewed the accessible data and, based on their analysis, arrive at the following conclusions regarding the current state of publicly available information:

The diversity of emerging gene therapy technologies, combined with the limited availability of comprehensive data, highlights the need for continued research and publication of results to support informed risk assessment and regulatory decision-making.

A key observation is the scarcity of accessible, detailed data on the biodistribution of gene therapy vectors to gonadal tissues, particularly for non-viral systems still in early preclinical development. The wide diversity of delivery systems and therapy-specific parameters - such as administration route, duration, and dosage - further complicate risk evaluation, making case-by-case assessment essential.

Although reproductive organs are frequently included in non-target safety assessments, publicly available data remain fragmented and inconclusive. As the field progresses, there is a clear need for more systematic research, harmonized testing protocols, and greater transparency in data reporting. Establishing a regulatory framework that fosters consistency across jurisdictions could strengthen confidence in data sharing and support clearer expectations for data validation. This highlights the critical role of regulatory guidance documents, even as they strive to keep pace with rapid scientific advancements. Despite current limitations, the findings of this study may offer a useful starting point for developing a more structured and scientifically informed approach to germline risk assessment, as discussed below.

The biological, physiological, and anatomical characteristics of the reproductive systems function as intrinsic protective barriers to germline exposure, thereby emphasizing the need for a thorough understanding of these mechanisms when evaluating the potential for germline exposure.

In males, the BTB–supported by the structural and functional roles of Sertoli cells - creates a highly selective and immune-privileged environment that limits the entry of exogenous genetic material. While certain viruses, such as ZIKV and HIV, can exploit vulnerabilities in this barrier under inflammatory or pathological conditions, therapeutic vectors generally lack such invasive capabilities. Preclinical studies involving exosomes, ferritin-based nanocarriers, and engineered lipid nanoparticles have shown potential to traverse the BTB and reach spermatogonial stem cells; however, these findings predominantly stem from non-systemic administration routes, such as intratesticular injection, limiting their relevance to standard gene therapy applications. Importantly, no heritable germline modifications have been documented in these studies. Published data indicate that viral vectors - particularly AAV - are occasionally detected in bodily fluids such as semen; however, they generally do not integrate into spermatozoa. Most evidence suggests that these vectors persist as episomal DNA and remain confined to somatic tissues when used within therapeutic parameters.

In females, the BFB surrounding the oocyte serves as a highly selective filter, effectively restricting the passage of large molecules and pathogens. Studies involving non-viral vectors - such as gold nanoparticles and lipid-based systems - have reported localization to peripheral ovarian structures, including the theca and stromal layers; however, no direct evidence of systemic delivery reaching the oocyte has been documented. Limited gene transfer into ovarian tissue has been observed following direct ovarian injection with AAV serotype 9. Nonetheless, due to study limitations, definitive conclusions regarding germline involvement cannot be drawn. Importantly, no transgene material was detected in the offspring, suggesting the absence of heritable genetic modification.

Taken together, the evidence indicates that germline exposure is strongly influenced by variables such as dosage, route of administration, vector design and surface modifications, biological barrier permeability, and local tissue inflammation. Many non-viral delivery systems exhibit pronounced hepatic tropism and are rapidly cleared from circulation, thereby limiting their interaction with reproductive tissues following systemic administration. Some preclinical studies have demonstrated that both viral and non-viral vectors can reach testicular or ovarian tissues when directly administered to reproductive organs. While these findings contribute to understanding the full biodistribution potential of these platforms, they reflect experimental conditions that differ from intended clinical use and should be interpreted accordingly. To date, ***no study has provided conclusive evidence of germline transmission or heritable genetic modification*** resulting from such exposures.

As gene therapy technologies continue to diversify, biodistribution assessment strategies must be adapted to remain relevant and scientifically meaningful.

To ensure relevance and reliability, biodistribution studies must be designed and interpreted in a way that meaningfully reflects the biological context and therapeutic intent of each gene therapy platform. The product-specific nature of biodistribution assessments may already differ between viral and non-viral delivery systems.

Viral vectors, due to their potential for genomic integration or stable episomal persistence, allow for longer-term tracking of the genetic cargo. In such cases, the detection of nucleic acid material can provide meaningful insights into vector biodistribution and, potentially, transgene expression. In contrast, non-viral systems typically induce a rapid but transient expression profile, necessitating the use of highly sensitive detection methods to capture relevant data within a narrow temporal window. Alternative techniques such as bioluminescence and fluorescence labelling may be employed to track the biodistribution of non-viral delivery systems in non-clinical studies.

However, these methods typically do not provide information on the biological activity of the introduced transgene. This limitation is particularly relevant in the context of gene-editing applications, where the absence of detectable editing components - delivered as DNA, mRNA, or protein - does not allow conclusions regarding the presence or absence of genomic edits. Although gene-editing agents are rapidly degraded and act within a narrow time window, the resulting genetic modifications - such as insertions, deletions, or base substitutions - are stable and permanent at the DNA level. Therefore, in assessing the risk of inadvertent germline modification, especially for non-viral delivery of gene-editing components, the initial steps of the tiered approach outlined in EMEA/273974/2005 are only meaningful if biodistribution monitoring techniques are sufficiently robust and sensitive within the early post-administration detection window. In cases where accurate mapping is not feasible, complementary approaches, such as deep sequencing of germline tissues or sequencing of off-spring, should be considered alongside biodistribution studies.

Data confirming the presence of gene therapy components in association with gonadal tissues are an important indicator. However, their absence must be interpreted with caution, as it does not definitively rule out potential exposure.

As previously noted, although theoretical pathways for germline modification exist, no documented cases have been reported in either preclinical models or human clinical studies involving gene therapy. However, it is fundamentally impossible to prove the absolute absence of such events, particularly in systems characterized by stochastic behaviour or extremely low-probability biological outcomes. As discussed above in the context of biodistribution studies, even when vector material is not detected in reproductive tissues, such findings must be interpreted with caution. While these data can inform estimates of germline exposure likelihood, the inherent limitations in the sensitivity and specificity of current analytical methods mean that negative results cannot be regarded as definitive evidence of absence.

Establishing methodological standards and clearly defined thresholds for detection sensitivity and specificity could enhance the reliability and reproducibility of biodistribution data, reduce inconsistencies in reporting formats, and support more robust risk-based assessments. However, current regulatory guidance related to inadvertent germline modification generally lacks such detailed criteria. Instead, existing frameworks are grounded in broad principles, aiming to balance the promotion of innovation and flexibility for platform-specific characteristics and novel

methodologies with the need for proportionate risk evaluation. This includes consideration of target populations and appropriate risk mitigation strategies. While regulatory guidance plays a critical role, maintaining its relevance in the context of rapid scientific advancement remains a challenge.

Any detection limit established for biodistribution studies must be evaluated not only in terms of analytical performance but also for its biological relevance. In the absence of a clear understanding of the threshold levels required to induce unintended germline modification, it remains difficult to determine the significance of detected - or undetected - vector material in reproductive tissues. Consequently, biodistribution data should be interpreted as indicative rather than definitive, particularly when no signal is observed. Without defined biological thresholds, the relevance of such findings to germline risk assessment remains uncertain, underscoring the need for continued research to bridge the gap between analytical sensitivity and biological impact.

Risk assessment for germline modification can be effectively based on biodistribution data and the specific attributes of the gene therapy strategy, enabling a targeted evaluation of safety and potential heritable effects

Current guidance documents strongly rely on biodistribution data under the premise that if the gene therapy component does not reach gonadal tissue, the risk of germline modification can be reasonably excluded. This approach reflects a mechanistic understanding that physical absence of the therapeutic vector or transgene from reproductive organs significantly reduces the likelihood of unintended heritable genetic changes, thereby serving as a foundational criterion in regulatory risk assessments. Notwithstanding the limitations and challenges associated with non-viral delivery systems discussed earlier, this remains a pragmatic and scientifically grounded step in evaluating germline safety.

As gene therapy technologies continue to evolve, particularly with the emergence of hybrid systems that combine viral and non-viral elements or employ fusogenic mechanisms, ongoing vigilance in biodistribution monitoring remains essential. Future advances in this field will likely depend on the development of improved imaging and molecular tracking technologies capable of detecting low-frequency germline interactions. Notably, both, the recognition of the continuous challenge of translating animal data to human physiological processes and the high-level incentives to replace, reduce, refine the use of animal models may potentially encourage the development of next approach methodologies.

A second element in the risk assessment involves the specific attributes of the gene therapy technology itself. Two major hazard factors inherent to the gene therapy product can affect the risk of germline modification : 1) the ability of a vector to integrate into host cell chromosomes and 2) the inclusion of gene-editing components as payload/cargo.

When the payload/cargo does not include gene-editing components, delivery vectors can be ranked by their relative ability to modify (to integrate) the germline genome :

- Vectors that enter into the cell nucleus and that carry an integration machinery
  These vectors can enter the cell nucleus and actively integrate into host chromosomes.
  - Viral vectors : e.g. retroviral vector, lentiviral vector
  - Non-viral systems : e.g. synthetic transposon systems (sleeping beauty, PiggyBac, with DNA cargo delivered by electroporation or lipid-based carriers); phage-derived and integrase-mediated recombination systems (such as PhiC31 and Bxb1 integrase).

Non-viral vectors are generally less efficient at nuclear entry and chromosomal integration compared to viral vectors. Of note, vectors that carry an integration machinery and that have advanced into clinical application mostly, if not all, concern *ex vivo* gene therapy applications (e.g. CAR-T cell therapy or *ex vivo* genetic modification of haematopoietic stem ad progenitor cells).

- Vectors that enter into the cell nucleus, without integration machinery
  These vectors can access the nucleus but do not inherently integrate into host chromosomes
  - Viral vectors : e.g. adeno-associated viral vectors, non-integrating lentiviral vectors
  - Non-viral vectors : e.g. polymeric systems loaded with nuclear localization signals (for promoting nuclear entry of DNA cargo)

- Vectors that are unable to enter the target cell nucleus and remain cytoplasmic
  These vectors do not enter the nucleus and remain confined to the cytoplasm
  - Viral vectors : alphavirus-based vectors, Sendai-based viral vectors, vesicular stomatitis virus-based vectors, rabies-based virus, MVA viral vectors, …
  - Non-viral vectors : liposomes, LNP, exosomes, …

From the ranking above, the extent to which vectors can access the nucleus increases the likelihood of direct germline genome modification. However, the ranking above necessitates a reconsideration when the payload/cargo includes gene-editing components (e.g. ZFN, TALEN, CRISPR-Cas9, base editors, prime editors, ARCUS nuclease). This is because transient cytoplasmic delivery of gene-editing components can result in stable and permanent modification of the germline due to the intrinsic property of gene editors to act on nuclear DNA.

Another aspect that requires consideration in the assessment of germline modification is the inherent biodistribution and host cell tropism, including the modification of viral vectors or the incorporation of targeting molecules into non-viral vectors to enhance delivery to specific tissues or organs.
Finally, an additional property inherent to the gene therapy product to consider in the risk assessment includes the durability of exposure. Prolonged persistence or activity of vectors or payloads can increase the risk of germline modification. For example, adeno-associated viral vectors, which is a workhorse for human gene therapy, have been made replication incompetent. Regarding the delivery of gene-editing

components, the delivery as mRNA and ribonucleoprotein complexes rather than DNA is expected to reduce the time for exposure.

While hypothetical risks associated with germline modification must be carefully considered, they should be weighed against the potential therapeutic benefits for patients, ensuring that safety concerns do not unduly hinder access to promising and potentially life-changing treatments.

In conclusion, although there is currently no evidence supporting the occurrence of unintended germline modification following somatic gene therapy, this absence of evidence does not equate to the absence of risk. Given the rapid pace of technological innovation and the inherent complexity of the biological systems involved, a precautionary yet adaptable regulatory approach remains essential. Strengthening methodological standards, improving data availability, and acknowledging the intrinsic limitations of current detection methods are all critical steps to safeguard both safety and public trust in gene therapy interventions.

Balancing hypothetical risks with potential patient benefits is a central challenge in the development and regulation of gene therapy technologies, particularly those with germline modification potential. While it is essential to rigorously assess and mitigate any theoretical risks - especially those with long-term or heritable implications - this must be weighed against the transformative impact these therapies can have on individuals suffering from serious or life-threatening conditions. A risk-benefit framework that acknowledges scientific uncertainty while prioritizing patient outcomes allows for responsible innovation without unnecessarily delaying access to promising treatments. This balance is crucial to ensure ethical progress and maintain public trust in emerging genetic technologies.

As a cautious and responsible approach to managing potential germline risks, mitigating measures such as reproduction control can be considered on a case-by-case basis. These measures not only help minimize the likelihood of heritable genetic changes but also provide a valuable opportunity to collect additional data that can inform future risk assessments and regulatory decisions. To support such decisions, a new flowchart (**Figure 4**) is proposed updating the original EMA framework (2006)[33] and providing a more flexible, tiered, and risk-based structure. It moves beyond a rigid binary model by distinguishing between early- and late-stage development, incorporating new technologies such as gene editing, and providing guidance on when risks can be considered negligible or when further evaluation is warranted. In this way, the flowchart complements the chapters of this report and serves as a practical tool for guiding risk–benefit evaluation. Furthermore, the use of platform technologies - characterized by consistent delivery systems and predictable behaviour across multiple applications - can streamline safety evaluations and potentially reduce the extent of data requirements for each new therapy. Together, these strategies support a balanced pathway for advancing somatic gene therapy while maintaining rigorous safety standards.

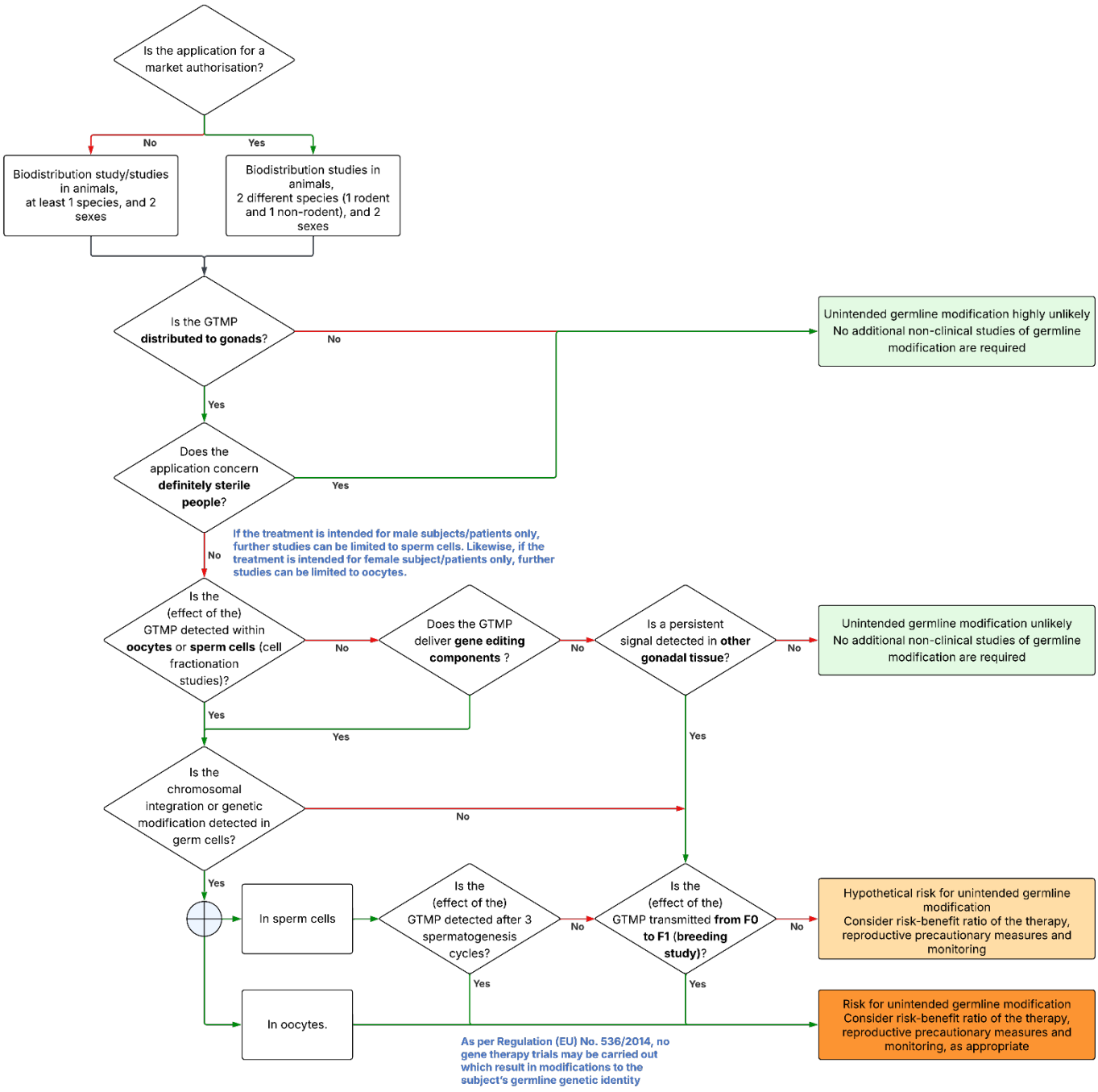

Figure 4. Proposed flowchart for the non-clinical assessment of potential germline transmission in somatic gene therapy medicinal products. *This flowchart provides a tiered, risk-based framework to guide evaluation of inadvertent germline modification. Compared with the original EMA (2006) version, it introduces greater flexibility by distinguishing between early- and late-stage development, integrating considerations for emerging technologies such as gene editing, and allowing for proportionate regulatory decisions beyond a binary prohibition model. The flowchart complements the discussion in this report by offering a practical tool for balancing hypothetical risks with potential patient benefits in the development and regulation of gene therapy.*

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8 Annex - Methods

8.1 Literature surveys

8.1.1 Background information

The information for the introductory parts of this report was obtained from recent reviews on the topic. Google Scholar was used, with a focus on highly cited publications.

8.1.2 Database Selection and Rationale

This project aimed to compile an overview of available preclinical data on various delivery systems for gene therapy, including viral vectors, liposomes, lipid nanoparticles, polymers, and viral-like particles. Particular attention was given to the biodistribution to gonadal tissue and germ cells. Additionally, the potential for germline modification was investigated, considering the characteristics of the delivery systems and the germline barrier.

To thoroughly evaluate preclinical data on gene therapy delivery systems, a structured literature search was conducted across multiple biomedical and interdisciplinary databases. The selected databases were PubMed, Embase, and Web of Science, chosen for their extensive coverage of peer-reviewed biomedical literature and indexing capabilities.

- **PubMed**: A premier database managed by the US National Library of Medicine, PubMed provides access to MEDLINE, a widely recognized repository of biomedical literature. It employs Medical Subject Headings (MeSH) for standardized indexing, ensuring precise and consistent retrieval of relevant studies.
- **Embase**: Maintained by Elsevier, Embase complements PubMed with a broader European and pharmaceutical focus. It employs Emtree indexing, similar to MeSH, but with additional keywords capturing emerging trends and pharmacological data.
- **Web of Science**: A citation-based multidisciplinary database that includes scientific literature across various domains. It does not rely on controlled vocabulary like MeSH but allows for comprehensive keyword-based searches, capturing relevant publications not indexed in PubMed or Embase.

By utilizing these three databases, we maximized the breadth and depth of our search, ensuring a comprehensive dataset for analysis.

8.1.3 Search Strategy Development

MeSH terms are standardized keywords used in biomedical literature to facilitate precise and efficient indexing, searching, and retrieval of information across various databases. In this study, the following MeSH Terms were used:

- Genetic Therapy
- Gene Transfer Techniques
- Liposomes
- Polymers
- Neoplasms, Gonadal Tissue
- Germ cells
- Drug evaluation, preclinical

Some databases rather use keywords, and the following were combined:

- Gene therapy delivery systems
- Preclinical data on gene therapy
- Biodistribution to gonads
- (Inadvertent) Germline modification
- (Inadvertent) Germline transmission / Germ-line transmission / Germ line transmission
- Vertical transmission (germline transmission is a type of vertical transmission)
- Gene therapy safety
- Lipid-based delivery systems
- Germline barriers
- Targeting germ cells
- (Non-)viral gene delivery
- (Non-)viral gene therapy
- Biodistribution
- DART

8.1.4 Search Strings and Selection

The search queries were customized for each database using the appropriate syntax.

Query	Search string	Results
PubMed	("Gene Therapy"[MeSH] OR "Gene Therapy"[All Fields] OR "Gene Transfer"[All Fields]) AND ("Viral Vectors"[MeSH] OR "Viral Vector"[All Fields] OR "Liposomes"[MeSH] OR "Lipid Nanoparticles"[All Fields] OR "Polymers"[MeSH] OR "Viral-Like Particles"[All Fields]) AND ("Biodistribution"[MeSH] OR "Biodistribution"[All Fields] OR "Gonadal Tissue"[MeSH] OR "Germ Cells"[MeSH] OR "Germline"[All Fields]) AND ("Preclinical Studies"[MeSH] OR "Pharmacokinetics"[MeSH] OR "Drug Delivery Systems"[MeSH] OR "Drug Evaluation, Preclinical"[MeSH])	142
Embase	('gene therapy' OR 'gene transfer') AND ('viral vectors' OR 'viral vector' OR 'liposomes' OR 'lipid nanoparticles' OR 'polymers' OR 'viral-like particles') AND ('biodistribution' OR 'gonadal tissue' OR 'germ cells' OR 'germline') AND ('preclinical studies' OR 'pharmacokinetics' OR 'drug delivery systems' OR 'drug evaluation, preclinical')	85
Web Of Science	ALL=(('gene therapy' OR 'gene transfer') AND ('viral vectors' OR 'viral vector' OR 'liposomes' OR 'lipid nanoparticles' OR 'polymers' OR 'viral-like particles') AND ('biodistribution' OR 'gonadal tissue' OR 'germ cells' OR 'germline') AND ('preclinical studies' OR 'pharmacokinetics' OR 'drug delivery systems' OR 'drug evaluation, preclinical'))	331

Following the execution of searches across databases, results were compiled and duplicates removed resulting in a selection of 460 unique publications. Of these, full texts were obtained for 440 papers were obtained. This structured and comprehensive search strategy enabled the systematic retrieval of relevant literature on preclinical gene therapy delivery systems, with particular attention to biodistribution to gonadal tissues and potential germline modification risks. The inclusion of multiple databases ensured a broad and diverse dataset, while the combined use of MeSH terms and keywords optimized retrieval accuracy and relevance.

8.1.5 Artificial intelligence analysis of literature search results

Selected studies were screened using AI-assisted data extraction. For this, the tool Elicit PRO was used to facilitate systematic review and annotation. A customized data extraction table was created to categorize information from 388 full-text PDF that included primary data (excluding reviews) across several aspects, including type of delivery system, research setting (in vitro, ex vivo, or in vivo), evidence of clinical application, presence of biodistribution data, detection methods employed, and evaluation of potential effects on germline cells. Each study was annotated accordingly upon submission of the full-text PDF versions of the selected publications. All extracted data were subsequently reviewed and verified manually to ensure accuracy and consistency.

8.2 Research projects

To assess whether and how ongoing research efforts and projects related to the development of novel gene therapies consider potential unintended germline modification, a targeted screening was conducted using three platforms allowing browsing of funded research projects. Information was accessed from CORDIS, focusing on Horizon Europe and past Framework Programmes, and ERIS (European research information system) which merely targets EU international coordination and monitoring. Because both focus on EU-funded research, another major international funding organization, the NIH RePORTER was used to cover US federally funded biomedical research. Information was accessed in March 2025, based on the following search strings and keywords:

Cordis	gene therapy' AND 'biodistribution' AND 'biological barriers' 'Non-viral vector' AND 'gene editing' AND '*in vivo*' 'Non-viral vector' AND 'germline' 'Non-viral vector' AND 'germ cells' 'gene editing' AND 'non-viral delivery
ERIS	'genome editing' AND 'gonad' 'genome editing' AND '*in vivo*' 'genome editing' AND ' *in vivo* gene therapy' 'genome editing' AND '*in vivo*' NOT 'plants' AND 'therapy'
NIH	'gene therapy' AND 'germline' NOT 'infertility' 'genome editing' AND 'delivery' AND ('gonads' OR 'gonadal tissue') 'genome editing' AND '*in vivo*' AND 'safety' AND ('gonads' OR 'gonadal tissue')

8.3 Expert inquiry

To support a comprehensive evaluation of unintended germline modification risks associated with *in vivo* gene therapy, a structured inquiry was conducted targeting external experts across key sectors. The objective was to identify current trends, challenges, and potential knowledge gaps within the regulatory, academic, and clinical landscapes.

For this, a semi-structured questionnaire was developed to guide the inquiry. The instrument was designed to capture expert insights across three main domains:

- respondent experience with assessing viral and non-viral vector delivery systems for in vivo gene therapy

- non-clinical data requirements and limitations for novel gene therapy approaches
- considerations related to clinical trial data and associated regulatory challenges. Specific attention was given to gene replacement and gene editing strategies, including base and prime editing.

Participants were purposively selected to represent a range of perspectives, including regulatory officials, academic researchers, and clinical investigators with recognized expertise in gene therapy. Potential contributors were contacted via email and informed of the study's aims, ethical considerations, and confidentiality measures. In addition a broad call for contributions was launched via the European Biosafety Association (EBSA) and the Association for Responsible Research and Innovation in Genome Editing (ARRIGE).

Participants were assured that all responses would be anonymized and de-identified, and that individual or institutional affiliations would not be disclosed.

9 Annex – Authors

This project succeeded through the strong collaboration between Perseus BV, a 3BIO Company, and the Service for Biosafety and Biotechnology (SBB) at Sciensano, uniting insights on biotech innovation with biosafety and regulatory expertise.

Perseus BV, founded in 2003, specializes in biosafety and biotechnology legislation, with a primary focus on Europe. The company works across sectors that utilize biological processes, offering services such as regulatory analysis, risk assessment, infrastructure and protective equipment evaluation, preparation and support for applications involving contained use and deliberate release of GMOs, training, and auditing. Its clients include biotech startups, multinational companies in the pharmaceutical, agrochemical, and petrochemical industries, academic institutions, government bodies, and NGOs. Since 2023, Perseus has closely collaborated with ABS-int BV, experts in Access and Benefit Sharing (ABS) of genetic resources, under the joint initiative 3BIO covering Biodiversity, Biosafety and Biotech Regulatory.

https://3bio.eu/

The SBB is part of the scientific directorate "Biological Health Risks" at Sciensano, Belgium's national public health institute. Sciensano supports public health policy through scientific research and expert advice, offering recommendations and solutions for proactive health strategies at national, European, and international levels. Established in 1995, the SBB is a multidisciplinary team with broad expertise in biosafety. It focuses on risk evaluation and, where appropriate, proposes proportionate risk management measures related to the use of pathogens or genetically modified organisms. The SBB is committed to providing scientific support, objective information, and recommendations to governments and other stakeholders, maintaining independence in its expert assessments - consistent with Sciensano's mission.

https://www.biosafety.be/