



289th ENMC international workshop: assessing and managing emerging AAV related toxicities after gene therapy for neuromuscular disorders, 26 - 28 September 2025, Hoofddorp, The Netherlands

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ARTICLE INFO

Keywords

Adeno-associated virus (AAV)
Gene therapy
Immune toxicities
Anti-transgene immunity
Innate immune response
Adaptive immune response
Endothelial injury

ABSTRACT

Adeno-associated virus (AAV) mediated gene therapies has emerged as a potentially transformative treatment approaches for neuromuscular disorders, with two FDA-approved products now in widespread clinical use: onasemnogene abeparvovec (Zolgensma) for spinal muscular atrophy and delandistrogene moxeparvovec-rokl (Elevidys) for Duchenne Muscular Dystrophy. However, severe and occasionally fatal adverse events affecting vital organs, including the blood, liver, muscle, and heart, have emerged in both clinical trials and real-world post marketing settings. The 289th European NeuroMuscular Centre (ENMC) workshop convened 38 participants from patient advocacy groups, industry, and preclinical and clinical research groups to collaboratively review these toxicities, their underlying mechanisms, and potential mitigation and monitoring strategies. Discussions addressed the clinical spectrum and biological drivers of these events, the respective roles of innate and adaptive immunity, the contribution of specific vector characteristics as well as of the specific disease and recipient. The application of risk stratification and immunosuppressive regimens for prevention, monitoring, and management were considered. Emerging toxicities, including capillary leak syndrome, endothelial and dorsal root ganglia injuries, were reviewed alongside corresponding preclinical data from non-human primates. Participants agreed on the need to harmonize standard operating procedures, clinical guidelines, and data-sharing practices, and endorsed collaborative initiatives to proactively address critical gaps and unresolved key questions through a patient-centered framework.

1. Introduction and overview

Chairs: Carsten Bönnemann, Hildegard Büning, Francesco Muntoni

The 2nd ENMC International Workshop on Gene Therapy Safety was conceived as an interactive forum to address established and emerging safety concerns associated with adeno-associated virus (AAV) gene therapies. The meeting aimed to advance a transparent and coordinated framework to proactively understand, monitor and manage toxicities

observed in clinical and post-marketing cases.

The organizers established the following key objectives:

- Review current toxicity landscape, including established, emerging, and anticipated toxicities associated with AAV gene therapies.
- Establish mechanistic causes of toxicities where possible and identify gaps in existing data and fundamental knowledge that need to be filled to clearly establish such mechanisms.

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- Propose standardized protocols for patient monitoring, comparative data collection, and biological sample acquisition, applicable both at a minimal achievable level and at a more complex, research-oriented level.
- Review current clinical practices for prevention and reactive intervention of toxicities, define areas of uncertainty, and establish a consensus on current best practices.
- Define determinants of patient susceptibility to AAV vector-based gene therapy, considering disease stage, co-morbidities, and other intrinsic factors.
- Enhance data sharing and communication across stakeholders and community.

The workshop brought together a group of multidisciplinary experts, from academic research, industry development, frontline clinical care, and patient advocacy. Regulatory agency representatives from the European Medicines Agency (EMA) and the U.S. Food and Drug Administration (FDA) were unable to attend due to restrictions by the corresponding agencies. The complete list of the ENMC workshop participants is included at the end of the report.

1.1. Historical Context: Summary of previous NIH, MDA and ENMC workshops on AAV safety

Francesco Muntoni, Sharon Hesterlee, Carsten Bönnemann

This 289 ENMC workshop builds on prior collaborative efforts that have progressively refined the key questions and challenges facing the field to advance the safety and efficacy of gene therapy.

ENMC 2022 Workshop [1]: This foundational meeting discussed the emerging safety landscape. Key discussion points included the significant limitations of preclinical rodent and non-human primate animal models, often lacking the underlying disease pathology of patients. The group also addressed regulatory challenges and began to explore the nascent understanding of specific toxicities and the role of individual patient susceptibility.

Muscular Dystrophy Association (MDA) Annual Gene Therapy Summit Meetings [2,3]: This series of four meetings has shown a clear evolution in focus. The initial session in 2022 centered on framing the overall landscape of toxicity. Subsequent meetings broadened in scope to include other conditions and a wider array of toxicities, more recently focusing on proactive mitigation strategies, developing predictive immune assays, and establishing clinical site readiness. The most recent meeting incorporated real-world evidence from post-approval rollouts. These meetings contributed to the identification of several "remaining gaps and opportunities," including the feasibility of redosing, managing late-onset toxicities, ensuring data transparency, defining long-term follow-up obligations, and aligning on global regulatory expectations.

National Institutes of Health (NIH) preparatory meeting in 2024: Its primary purpose was to build on the momentum of a prior industry-investigator working group [4] to expand and formally establish a working group structure that would prepare and guide the present discussions. The NINDS director, Walter Koroshetz, cautioned against the impulse to prematurely abandon current AAV technologies that are already in the clinic in favor of future AAV innovations as safety of such novel vectors needs to clearly demonstrate a benefit versus the better known "first generation", and this cannot be taken for granted. He stressed the collective responsibility to improve and secure the safety of the therapies that are already in the clinic today, emphasizing the need to "make sure that what we have currently is getting better and safer" rather than ignoring the problems at hand.

Pre-ENMC virtual working groups: Prior to the in-person second ENMC meeting, four working groups met virtually to start discussions on the pressing clinical and research questions related to transgene, liver, cardiac, and TMA toxicities. Acknowledgment of all participants who contributed to these virtual discussions is provided at the end of the report.

1.2. Patients and advocates perspectives

Elizabeth Vroom, Pat Furlong, Sharon Hesterlee

Patient advocacy representatives emphasized the challenge of managing the "hope and hype" cycle alongside confusing information. Families receive messages suggesting that a multi-million-dollar treatment is a definitive cure, creating false hope and immense pressure. This environment makes it difficult for parents to navigate complex decisions about pursuing gene therapy versus other available treatments. The specific challenges posed to the workshop attendees include:

- The urgent need for clear, consistent communication of the known and what remains unknown about the benefits and risks of gene therapy.
- Avoidance of misleading terminology, such as "cure" "transformative," and "life-changing," which are easily misinterpreted by families and create unrealistic expectations.
- The difficulty families face when trying to make informed decisions about combining therapies or timing gene therapy, often with incomplete information and the added complication of securing insurance coverage.

Advocates highlighted the significant variability in clinical care protocols between different treatment centres, which causes confusion and anxiety and leading families to question whether their child is receiving the "best" or "right" care. In response to the call for protocol standardization, clinicians noted that complete, rigid protocolization across all sites may not be feasible or even desirable. Care decisions, such as the requirement to remain in close proximity to a treatment centre, must often be tailored to a patient's individual circumstances, including their geographic location and the capabilities of their local healthcare resources. There was a strong consensus, however, that while protocols may differ, the rationale for those differences must be clearly articulated and communicated to families, as transparency is key to building trust and alleviating the anxiety caused by perceived inconsistencies. A forward-looking proposal was put forth to proactively establish community-enforced, outcomes-driven quality metrics. Drawing a parallel to highly regulated fields (such as cardiac surgery), where public reporting of outcomes drives quality improvement, this framework could offer a powerful mechanism to promote standardization and ensure site preparedness. By tracking and comparing outcomes (i.e. management of the adverse events) across a network of centres, best practices could be identified and disseminated. Such a system could be particularly impactful in a fragmented healthcare system, helping to ensure that all patients receive a high standard of care regardless of where they are treated.

Specific areas that were pointed out as requiring clarification and standardization include: post-treatment quarantine, physical proximity to clinical care site post dosing, with a clear distance definition and required timeframe, the definition of "ambulatory" in the setting of clinical trials for the purpose of determining eligibility or for drug labelling.

Significant public health concern was raised regarding AAV vector shedding and its potential to increase immunity in the broader community as more patients are dosed. Importantly, the FDA has issued guidance on this topic, which has shaped current sponsor practices. In addition, Sarepta presented data from over a thousand treated patients, showing that viral shedding declines to very low levels within two weeks post-dosing. A Pfizer study, though acknowledged to be very small and underpowered (n=8), systematically tested household members of dosed patients and found no evidence of seroconversion. While current data are reassuring, the consensus was that the field lacks definitive, sufficiently powered studies to conclusively address this issue, leaving a critical communication and public health gap that needs to be proactively addressed.

The profound physical and psychological burden of the mandated

high-dose steroid regimen was presented as a critical quality-of-life issue. The side effects of prolonged use of higher doses of steroids can represent a gruelling experience that dramatically impacts the entire family. Advocates shared their experience with severe behavioural changes including psychosis and noted the increased risk of bone fractures during this period. This highlighted a critical question for the assembled experts: Is this legacy steroid protocol, adopted because it is the established practice, truly the best approach? The challenge was issued to move beyond this method of immune modulation and explore more refined, targeted strategies that can achieve the desired therapeutic effect without such a disruptive impact on patients and families.

This discussion highlighted the vital partnership with patient organizations, to re-centred priorities, moving beyond a narrow focus on technical solutions to a holistic approach that encompasses clear communication, quality of life, and systemic care delivery. Patient advocacy group participants emphasized the need to explain to the patient community what is still unknown about gene therapies and their potential impact. This information helps patients and families better understand many dimensions of the process including their participation in trials, biopsy sampling and often complex consent forms. The absolute need to report relevant findings back to patients who provided samples and data was stressed. A consensus emerged that the gene therapy community must commit to sustained, proactive, and collaborative work through the established working groups to translating these

conversations into concrete actions, ensuring that the human impact of therapy is weighed as heavily as its biological benefit.

2. Anti-transgene immune reactions

Chairs: Serge Braun and Jeffrey Chamberlain

This session outlines the aggregate clinical experiences with immune-mediated myositis (IMM)/myocarditis, analyses the complex immunological data from T-cell and antibody assays, and details the discussions surrounding the management and diagnosis of these events and consolidates the key unanswered questions and actionable recommendations for the path forward.

2.1. Updates on transgene-related adverse events, presented by James Richardson (Sarepta), Serge Braun (Genethon), Qi Shen (Pfizer), Alexander Murphy (Roche), Carsten Bönnemann

2.1.1. Duchenne muscular dystrophy

Analysis of clinical trial and post-marketing data from Sarepta, Genethon and Pfizer reveals an emerging and fairly consistent but uncommon pattern of immune-mediated myositis (IMM) characterized by a specific constellation of risk factors, clinical presentations, and significant challenges in diagnosis and management. The discussion, centered on case studies from Sarepta Therapeutics (presented by James

Table 1
Comparison of the key patient cases with IMM.

Identifier	Genetics	Onset*	Key clinical features	Biomarkers	Outcome
Sarepta Therapeutics					
Patient 1 ¹	Exons 3-43 deletion	35	Dysphonia, dysphagia proximal muscle weakness, respiratory failure; no cardiac involvement	ELISpot for Pool 1 (exons 1-9) strongly positive at week 4. CD8+ T-cell infiltration targeted dystrophin-expressing myofibres on muscle biopsy	Recovered to baseline by day 84. Dystrophin expression below level of quantification post-event. Successfully weaned off immunosuppression
Patient 2 ¹	Exons 8-9 deletion	1st: 27 2nd: ~1-year later	Weakness, frequent falls, co-presenting with strep infection. The second episode included cardiac involvement and rhabdomyolysis	ELISpot for Pool 1 was positive at day 33 and became positive again during the second episode	The second episode occurred after the tacrolimus dose was reduced. Now on chronic immunosuppression (sirolimus, rituximab, monthly IVIG). Retained good microdystrophin expression on muscle biopsy
Patients 3-6 (post marketing reports of "possible" cases of myositis)	Deletion in exons 61-79; 12-25; 49-62; 10-19	between days 25 and 56 post-infusion	"Possible myositis," 2 of 4 patients showed bulbar symptoms; 2 of 4 with concurrent viral infection	Diagnosis was based on clinical presentation, timing, MRI reports (not images), and positive response to increased immunosuppression	n/a
Genethon ²					
Patient 1	In-frame exon 8-21 deletion (clinically classified as DMD)	21-28	Generalized weakness, bulbar signs	Rise in CK and troponin I; No micro-dystrophin detected at the 8-week biopsy, elevated IL-6 and IL-8 plasma cytokine levels at week 8; no follow up biopsies;	Did not respond to steroids or IVIG. Symptoms resolved after initiating tacrolimus. Recovered and weaned off immunosuppression after one year.
Pfizer ³					
Patient 1	Exons 8-43 deletion	25	Rhabdomyolysis	Extremely high micro-dystrophin ADA titre (>30,000 at 1 year) despite clinical recovery; ELISpot positive before GTx with no follow up results	Fully recovered by day 73. Remained clinically well despite persistently high microdystrophin ADA levels
Patient 2&3 (identical twins)	Exons 5-41 deletions	35	Muscle weakness and myocarditis	Micro-dystrophin ADA titres initially decreased with high-dose steroids but then spiked dramatically to very high levels years later. ELISpot positive from week 35 onward.	Myocarditis resolved by day 135; muscle weakness improved but not fully resolved.
Patient 4	Exons 55-59 deletion	45	myositis	Persistently high micro-dystrophin antibodies titres. ELISpot positive from week 35 onward; Biopsy at 1 year showed almost no transgene expression; micro-dystrophin antibodies titres remained high despite loss of transgene expression.	No significant clinical improvement despite extensive treatment (steroids, plasmapheresis, tacrolimus, rituximab).

* days post GT dosing

¹ Phase Ib, NCT04626674

² GNT0004

³ Phase 3, NCT04281485

Richardson), Genethon (presented by Serge Braun) and Pfizer (presented by Qi Shen), identified a distinct clinical syndrome of IMM in GT-treated DMD patients emerging from the collective clinical experience. Symptoms typically appear between 25 and 56 days post-infusion, often including bulbar signs (dysphonia, dysphagia) and progressive muscle weakness, with or without signs of myocarditis.

A comparison of the patient cases illustrates both the commonalities and the unique features that inform our current understanding of this toxicity, summarized in Table 1.

High-risk genotypes and exclusion criteria: A strong consensus is emerging that patients with specific DMD gene deletions, particularly those involving exons 8 and 9, are at a significantly higher risk for developing IMM [4,5]. These exons code for hinge 1, a region that, although present in utrophin, is shortened and relatively divergent from dystrophin due to a deletion and several point mutations, which may reduce the likelihood of inducing cross-tolerance. Analysis by three of the sponsors confirmed cytotoxic T cells reactive to peptides representing this region [4,5].

The transgene product-directed, immune-mediated myositis following gene delivery prompted all companies with ongoing DMD clinical trials to revise their eligibility criteria to exclude deletions affecting exons 8 and/or 9. Other regions, including exons 1-17 and 59-71, have also been identified as areas of potential concern, leading some sponsors to adopt additional genotype-specific exclusion criteria.

2.1.2. X-linked myotubular myopathy

A presentation on two CRIM-negative patients from the Astellas *MTM1* trial provides a relevant parallel for chronic anti-transgene immunity in the form of myocarditis-myositis. One of them, treated at 2.5 years of age, developed myocarditis (troponin peak) with a CK elevation (x10 of baseline) at 4 weeks post gene therapy. The patient was managed with sirolimus (rapamycin), and previously positive ELISpot results became negative. However, six years later, after weaning off sirolimus, the patient experienced a recurrence with a sharp troponin spike and a newly positive ELISpot against transgene peptides. Cardiac MRI confirmed a new focal area of myocardial inflammation, indicating an ongoing T cell mediated transgene directed immune response. There was no impairment of cardiac function and the patient retained significant clinical benefit from the therapy in terms of muscle and respiratory function.

2.2. Challenges in diagnosis of myositis and immune monitoring

Diagnosis of myositis and its differentiation from the underlying pathology, or other complications, for example exercise-induced rhabdomyolysis in DMD, remains a primary challenge particularly in the post-marketing setting. Although muscle biopsy can provide valuable mechanistic insight and is considered the gold standard for establishing the diagnosis, it is rarely performed in acute clinical settings, particularly when the patient is already responding to empiric immunosuppressive therapy. Moreover, the pathology should be examined by a neuropathologist with expertise in dystrophinopathy pathology, and in acquired myositis. Muscle MRI was identified as a helpful, non-invasive tool for detecting muscle inflammation. However, its broader utility is hampered by a lack of standardized imaging protocols and again expertise in interpreting findings that could overlap with the primary dystrophic pathology. The reliability of specific biomarkers remains debatable. Creatine kinase (CK) is a poor marker, as in several confirmed cases, CK levels were not remarkably elevated above the patient's elevated baseline or showed variability consistent with the natural history of DMD.

The workshop dedicated significant time to evaluating the clinical utility and inherent limitations of the primary tools currently available for immunological monitoring: the anti-drug antibody (ADA) assays for humoral responses and the enzyme-linked immunospot (ELISpot) assay for T-cell responses. The interpretation of both is complicated by high

variability, baseline positivity, and an unclear correlation with clinical events.

High-titer ADA responses are frequently observed across the trials, but their clinical significance is poorly understood. ADAs may be directed against either the delivery vehicle, such as anti-AAV antibodies, or the transgene product (TP antibodies) [6]. Extremely high micro-dystrophin antibodies titers are noted in patients with severe IMM, yet many patients with those high ADAs remain asymptomatic. While these assays provide invaluable research data, their role in routine clinical decision-making remains complex and uncertain.

Pfizer's data from their Phase 1 study showed that a majority of patients (50-60%) develop micro-dystrophin antibodies, with titers typically increasing over the first year. The average micro-dystrophin antibodies titers were low up to day 90 and steadily increased thereafter with the maximum average titers occurring at day 360. In the Phase 3 study, 63% of fordadistrogene movaparvec treated participants showed positive micro-dystrophin antibodies. However, this did not appear to affect the quantity of the transgene produced in those patients determined by muscle biopsy at baseline and after one year. A critical observation was that very high micro-dystrophin antibodies titers did not necessarily correlate with ongoing IMM clinical symptoms or prevent clinical recovery. This suggests the mere quantity of TP antibodies is not a definitive marker of destructive immunity. One concern emerging from the discussion was that antibodies may function as a "warning sign of pending rejection," even if not directly pathogenic themselves. To decipher the role of ADAs, experts agreed on the need to investigate antibody isotypes. This is based on the hypothesis that different immune pathways produce distinct isotypes; for instance, TH1 (cell-mediated) responses may correlate with an IgG1 subtype, while TH2 responses may correlate with IgG4. Understanding this could help stratify the risk associated with the ADA response.

Regarding ELISpot, high variability and background signal characterise this assay. It remains a valuable research tool but is not yet validated for guiding individual patient management decisions in the clinic. In Sarepta's clinical trial participants who developed IMM, positive ELISpot results in peptide pools covering high-risk epitopes (exons 1-9), correlated directly with the timing of the clinical events. In one case, the ELISpot became positive again upon clinical relapse, pointing to its potential as a marker of active T-cell-mediated rejection.

Data from Pfizer's Phase 3 study revealed an unexpectedly high rate of ELISpot positivity at baseline. Remarkably, 42% of patients in the gene therapy group and 48% of participants in the placebo group had a positive T-cell response at baseline, supporting the hypothesis that pre-existing T-cell responses may be directed against naturally occurring revertant dystrophin fibres in patients with DMD, as previously demonstrated in natural history studies [7,8].

2.3. Treatment and prophylaxis: current immunosuppressive strategies

In response to the emergence of IMM, sponsors and clinical investigators have progressively evolved their immunosuppressive protocols. The management of these events requires a three-pronged approach: aggressive intervention for acute episodes and a considered strategy for long-term control to prevent recurrence but also adjusted preventive protocols. Evidence from both DMD and MTM1 cases indicates that a subset of patients having experienced IMM/myocarditis may have a recurrence upon tapering immunosuppression. This suggests that tolerance is not always achieved and that some individuals may require long-term immunosuppressive therapy, akin to solid organ transplant management, to prevent chronic rejection and preserve transgene expression.

When a patient presents clinical signs of IMM, a rapid and robust immunosuppressive response is critical. The primary interventions used include high-dose corticosteroids, intravenous (IV) methylprednisolone, plasma exchange (plasmapheresis) and in some cases Intravenous immunoglobulin (IVIG) (though it was noted that the rationale for IVIG

use is unclear, as immunomodulation by blocking antibodies may not be acutely relevant to controlling anti-transgene immunity). For controlling the immune response long-term, calcineurin inhibitors and mTOR inhibitors have become the agents of choice. Tacrolimus was used effectively in both Sarepta and Genethon cases to gain control of IMM and facilitate recovery. Since then, Genethon has proactively adopted sirolimus prophylaxis into its standard protocol for all patients. The rationale is based on immunologic data suggesting sirolimus preferentially suppresses inflammatory T-effector cells without negatively impacting protective T-regulatory cells. It was emphasized that dosing for these agents must be managed by targeting therapeutic blood trough levels and not by a fixed daily milligram dose to account for high pharmacokinetic variability, age related differences in metabolism, and ensure effective immunosuppression. A significant unresolved issue is determining how long to continue therapy. The risk of recurrence of the myositis is real, as illustrated by the Sarepta patient who relapsed after discontinuing the immunosuppressive therapy 1 year after developing IMM. In a CRIM negative *MTM1* patient who developed an immune mediated myocarditis controlled by sirolimus, cessation of therapy after six years of stability led to a clinical and laboratory relapse providing definitive evidence of remarkably durable immune memory. Moreover, the acute, visible IMM cases may be the tip of a much larger, insidious threat, chronic transgene rejection. The Sarepta relapse upon weaning of immunosuppression and the *MTM1* relapse after six years all point to the risk of chronic, low-grade immune activity that potentially could silently erode long-term therapeutic durability.

2.4. Fatal Recurrent Rhabdomyolysis in a DMD Patient

The fatal DMD case reported by Qi Shen presents a complex challenge. The patient from the DAYLIGHT study was a 3-year-old boy diagnosed with DMD based on clinical phenotype, but with a 45-48 in-frame exon deletion, typically associated with the milder Becker muscular dystrophy (BMD). The patient received micro-dystrophin gene therapy at a dose of 2×10^{14} vg/kg and initially responded well, with a drop in CK and improved motor function. Seven weeks post-dosing, he experienced the first of 17 episodes of severe muscle pain and rhabdomyolysis, with extremely high CK levels. These episodes were managed with high-dose steroids, however remission period between events progressively shortened from ~60 days to less than three weeks. The last rhabdomyolysis episode culminated in a fatal cardiac arrest from hyperkalemia. Investigations revealed low-to-negative anti-dystrophin antibodies, distinguishing it from previously characterized immune-mediated myositis cases. A muscle biopsy taken after recovery from the first episode showed apparent full length dystrophin expression (estimated at 25-50% of control levels) with robust expression of transgene product and no significant inflammatory myositis. A second muscle biopsy, taken after one year of gene therapy dosing and soon after an acute episode, before treatment with steroids, did not show evidence of myositis or inflammation. There were minor myopathic features, such as minor active necrosis and regeneration and variation in fibre size. An exploratory ELISpot assay with cells collected between episodes of pain (6 months after onset) identified an Interleukin-17 (IL-17) T-cell response (but not IFN-gamma response) to regions of the patient's endogenous dystrophin, but not to the micro-dystrophin transgene product. However, biopsies did not show overt cellular infiltration to the muscle. During the 17th episode the patient experienced severe and fatal hyperkalemia, despite never presenting with elevated potassium in previous episodes. Autopsy revealed significant multifocal acute necrotising myopathy, with patchy regions of unaffected muscle, but no evidence of cardiac injury (nor in other organs).

There is no clear conclusion on this case, however potential mechanisms and risk factors were discussed. A few severe BMD patients with in-frame DMD deletions have received AAV GT in the real world but did not present a similar reaction. This case is unusual and may have been caused by a combination of factors, including a heterozygous missense

DAG1 variant of unknown significance (VUS), as mutations in this gene have been linked to rhabdomyolysis [9], combined with dystrophinopathy weakening the sarcolemma and lowering the threshold for muscle injury. This remains highly speculative. The lack of micro-dystrophin antibodies but presence of an IL-17 response to endogenous protein suggests the possibility of an atypical immune response via a T-cell mediated mechanism that is not fully understood. The recurrent, almost cyclical nature of the rhabdomyolysis is highly unusual and lacks a clear biological explanation. The absence of clear triggers (e.g., infection, exercise) further complicates the understanding of the driving mechanism. The autopsy, which revealed extensive necrosis, suggests the pre-fatal biopsies may not have captured the full extent of the pathology which nevertheless was acute and cyclical in this patient. A suggested strategy was to re-examine all available tissue samples with advanced techniques to better characterize any cellular infiltrates (e.g., HLA staining) and assess the co-localization of microdystrophin expression with cellular stress markers.

2.5. Actionable recommendations

To address unanswered questions related to the monitoring and management of transgene associated toxicities, the following actionable recommendations were proposed: (1) Champion the development and broad adoption of standardized muscle MRI protocols for both baseline and post-treatment assessment. (2) Refine the IMM case definition, including criteria for "possible," "probable," and "confirmed" cases to standardize safety reporting and improve pharmacovigilance. (3) Develop and validate better biomarkers, to create clinically validated, commercially accessible assays for monitoring both T-cell and antibody responses. (4) Establish a collaborative framework or registry for systematically collecting deep clinical, imaging, and biological data from post-market patients to create a robust dataset needed to better understand rare events like IMM.

3. Thrombotic microangiopathy and innate immunity in AAV gene therapy

Chairs: Genevieve Laforet and Kevin Flanigan

3.1. Definition and clinical presentation

Thrombotic microangiopathy (TMA) is a significant and serious adverse event observed in the context of high dose AAV gene therapy. It is a clinical phenomenon involving the formation of clots in the microvasculature, leading to a characteristic triad of clinical findings: (1) Microangiopathic hemolytic anemia: mesh-like clots shred red blood cells as they pass through the vasculature, creating fragmented cells (schistocytes) visible on a peripheral blood smear. (2) Thrombocytopenia: platelets are consumed in the formation of microthrombi, leading to a clinically significant decline in platelet counts. (3) End-organ Injury: The circulatory compromise caused by the clotting events most classically manifests as acute renal injury, indicated by hypertension and elevated creatinine [10]. Other organs can also be affected. Two key clinical cases were presented to illustrate the range of severity and clinical presentation of AAV-associated TMA.

Case Study 1: Severe, fatal TMA in an SMA patient [11]. This case, recapitulated by Genevieve Laforet, involved a four-month-old infant with SMA treated with onasemnogene abeparvovec (Zolgensma) under an expanded access program. Non-specific symptoms (lethargy, vomiting) and key laboratory signs (thrombocytopenia) appeared around day 8 post-dosing. The clinical presentation advanced to acute renal failure requiring hemodialysis along with hemolytic anemia, completing the classic TMA triad. Eculizumab (a C5 complement inhibitor) was first administered late in the course of TMA, on day 13. Despite initial signs of improvement, the TMA recurred in association with a hospital-acquired staph infection and sepsis. Additional eculizumab was

administered on Day 19 and 40. The patient ultimately succumbed approximately two months after gene therapy dosing due to hypovolemic shock, sepsis, and cardiac failure. Post-mortem genetic testing revealed a variant of uncertain significance (c.1322A>G; p. (Lys441Arg)) in the complement factor I (*CFI*) gene, a known inhibitor of the alternative complement pathway, suggesting a potential predisposing factor.

Case Study 2: Mild TMA with co-occurring cardiotoxicity in a DMD patient

This case, originally presented at ASGCT 2025 by Benjamin Samelson-Jones and shared by Kevin Flanigan, involved a 17-year-old ambulatory male with Duchenne muscular dystrophy (DMD) treated with delandistrogene moxeparvovec-rokl (Elevidys) in the post-marketing phase. The initial presentation was at days 2-4 with elevated troponin, EKG changes (T-wave inversions), a decline in left ventricular ejection fraction (from 63% to 52%), markedly elevated blood pressure, and atrial fibrillation. A cardiac MRI showed evidence of myocarditis- suggesting AAV-related myocardial inflammation. Following the acute cardiac events, within the first 10 days post gene transfer, the patient developed evidence of TMA, including: thrombocytopenia (platelets < 150,000/ μ L), increased complement activation (elevated soluble C5b-9), evidence of renal insufficiency (elevated cystatin C), presence of schistocytes on a peripheral blood smear though there was no evidence of anemia. The TMA in this case was considered mild and resolved without specific TMA-directed interventions. The clinical course suggested two distinct but potentially overlapping toxicities: an immediate cardiotoxicity and a subsequent TMA, and it remains unclear whether these two might be linked mechanistically via innate immunity pathways.

3.2. Post-AAV innate/inflammatory responses

Immune responses to the AAV vector are the primary drivers of the pathophysiology leading to TMA. This involves both the humoral innate system (complement) and the cellular innate system, which provides the initial inflammatory trigger [12–17]. The complement system is a cascade of proteins that, upon activation, converges to form the membrane attack complex (MAC), or C5b-9, which can insert into cell membranes and cause lysis. Activation of the complement system is mediated through 3 different pathways: (1) Classical pathway: strongly activated by antigen-antibody complexes, particularly those involving IgM, which is characteristic of a naive immune response. (2) Lectin pathway: recognizes polysaccharides on pathogen surfaces. (3) Alternative pathway: maintains a low level of "tonic" activity that can rapidly amplify upon encountering a foreign surface. All three pathways converge at the cleavage of C3, proceeding down a common pathway that generates C5b-9. When discussing the role of pre-existing antibodies, the absence of a universally accepted assay for antibody detection was highlighted. Antibody tests are typically developed as product-specific companion diagnostics, tailored to the individual gene therapy vector or platform. As a result, immunological assessments are highly sponsor-specific, limiting cross-trial comparability. Pre-existing antibodies are typically low-titer and low-affinity, and eligibility for treatment generally requires that antibody levels fall below a predefined threshold [18]. It cannot however be excluded that low levels of pre-existing antibodies could induce a faster rise of IgG with the re-exposure, therefore being a risk factor for TMA and other adaptive immunity triggered events. At the same time, it was noted that patients who are truly seronegative at baseline may mount a predominantly IgM response following exposure. IgM is highly efficient at activating complement via the classical pathway, leading to C3 cleavage and the formation of the membrane attack complex [19]. Differences between AAV serotypes were also highlighted as important factors; for example, AAV9 may be more effective at activating complement than AAV8 or AAVrh74, which may partly explain differences in immune activation observed between products.

Potential mechanisms by which antibodies may interfere with gene therapy efficacy relates to four principal mechanisms: (1) direct binding of antibodies to the vector, thereby preventing interaction with the target cellular receptor; (2) interference with cellular uptake, including effects on endocytosis; (3) intracellular effects that impair endosomal escape; and (4) potential downstream effects on vector uncoating and transgene expression. Regarding vector binding, in treated patients with low levels of pre-existing, binding antibodies to the vector, circulating antibodies were often undetectable as early as 1-hour post-infusion. This phenomenon is consistent with observations for other anti-drug antibodies, whether specific or nonspecific, which rapidly bind to their antigen and are subsequently cleared or trafficked within the reticulo-endothelial system. Despite this early binding, clinical efficacy of the gene therapy product is typically maintained, suggesting that the effect of antibodies at these low titers can be overcome by the high molar excess of vector administered relative to circulating antibodies. However, by approximately 72 hours post-infusion, antibody levels rise and isotypes begin to change, reflecting the development of an adaptive immune response and potentially altering vector clearance and immunogenicity profiles [20]. A central theme was that most available interventions are most effective when implemented pre-exposure - before vector administration - whereas post-exposure immune modulation is substantially more challenging and less predictable. Commonly used serology assays do not reliably distinguish between patients who are seronegative versus those who are truly immunologically naive. Only after vector exposure, when class switching becomes evident, can these states be differentiated with confidence. More granular approaches, including analysis of B-cell receptor rearrangements, were discussed as potential tools to infer prior exposure status and immune history.

3.3. Immunomodulatory strategies and administering AAV in patients with pre-existing immunity

Several immunomodulatory strategies to mitigate antibody-mediated early interference with gene therapy were discussed. Extensive preclinical data, together with substantial clinical experience in managing the immunogenicity of AAV vectors, are critical for successful clinical translation and for demonstrating safety and efficacy [21].

B- and T-cell-targeting approaches combining rituximab (anti-CD20) and sirolimus have been used, building on longstanding experience with anti-drug antibody management in enzyme replacement therapy and transplantation. The combination was found to be more effective than either agent alone. Rituximab targets CD20-expressing cells; however, CD20 expression is lost upon plasma cell differentiation. Because B cells are continuously generated, particularly in the setting of persistent antigen exposure, single-dose rituximab is insufficient to suppress emerging clones. Sirolimus contributes by modulating T-cell helper cells, thereby attenuating B-cell activation and potentiating the effect of CD20 depletion.

In a cohort of 38 patients treated across three studies (with different AAV products at different doses), outcomes were compared between those receiving conventional glucocorticoid-based immunosuppression and those treated with rituximab plus sirolimus [19]. Although antibody increases (IgM and IgG) were not completely prevented, particularly without a multi-dose regimen, combination therapy was associated with attenuated complement activation. Clinically, early complement activation was reflected by platelet decline, which was more pronounced in patients treated with corticosteroids alone. While some degree of microvascular injury was still observed, the combination regimen may have limited substantial antibody titer elevation and reduced activation of the classical complement pathway. The caveat is that differences in product dose may also have contributed to this observation, given the nonlinear, threshold-like relationship between dose and TMA-like events.

Because plasma cells lack CD20, additional approaches are required to address established humoral immunity. Strategies discussed included

targeting plasma cells directly and reducing circulating IgG through therapeutic plasma exchange or IgG-cleaving endopeptidases [22].

These methods may transiently lower antibody levels, although the durability and immunogenicity of the enzymes themselves remain limitations.

Another strategy involves modifying the AAV capsid to remove immunoreactive epitopes. Antibody-evading capsids have been developed. However, while these engineered capsids may reduce recognition by pre-existing antibodies, they do not eliminate the adaptive immune response following exposure.

Preclinical work explored whether alternative micro-dystrophin constructs or modified dosing strategies could overcome pre-existing immunity [23]. These nonclinical studies were designed to assess whether immune modulation combined with vector engineering or dose fractionation could improve transgene expression under conditions of pre-existing humoral immunity, with the potential for later clinical translation [24].

3.4. Innate sensing of AAV

Research using human iPSC-derived neural models and organoids reveals that the very first immune signals are generated within the target cells themselves, even before a systemic response occurs. This "cell-autonomous" sensing acts as the spark that can prime subsequent adaptive immune responses. There is a dual trigger mechanism – the first is activated through DNA damage response, the incoming AAV vector DNA, which is often derived from bacterial plasmids and lacks eukaryotic methylation patterns, is perceived by the cell as DNA damage activating the toll-like receptor 9 (TLR9)-MyD88 pathway [25]. This activates the P53 pathway, independent of transgene expression. The second is an inflammatory response to the expression of the transgene and the resulting RNA species activate pattern recognition receptors [26], triggering an inflammatory cascade that includes Type I interferon signalling through the adaptor molecule MAVS [25]. This response is dependent on transgene expression. In other words, while inflammation seems to be more closely linked to productive transgene expression, an un-transcribing vector genomes elicit mainly p53-dependent DNA damage response [25].

The quality of the vector preparation is critical. Contaminants such as partially filled capsids or small nucleic acid fragments attached to the capsid surface act as pathogen-associated molecular patterns (PAMPs) that can powerfully stimulate these innate pathways. Information on the potential contaminants of the vectors are not publicly disclosed although a recent manuscript assessed by long and short read meta-genomic analysis of onasemnogene abeparvovec identified residual manufacturing plasmid sequences [27]. An illustrative case of an SMA patient treated with onasemnogene abeparvovec provided important insights into potential toxicities related to vector integrity. This case, reported by Francesco Muntoni, is described in greater detail in the section on liver-related toxicities.

The role of pre-existing inflammation in SMA should also be considered. Using a mass spectrometry-based inflammasome panel (Muntoni et al, manuscript in preparation), it has been shown that SMA patients possess a significant baseline inflammatory profile before treatment. The severity of this pre-existing inflammation correlates with clinical disease severity, with Type 1 SMA patients exhibiting the most abnormal profiles. AAV gene therapy infusion was shown to further increase the inflammasome response [28]. Patients continued to show rising immunoglobulin production for up to eight months post-infusion. The patient who experienced a particularly severe adverse event also had the most significant rise in their inflammasome profile. Furthermore, SMA is associated with an underlying vascular pathology, including abnormalities in circulating endothelial cells [29]. This pre-existing condition may contribute to the immune toxicities variability in SMA patient in response to systemic AAV therapy.

3.5. Clinical experience, monitoring, and emerging safety concerns

Effective safety management requires aggressive and early monitoring that often exceeds label requirements. Consensus guidelines are emerging that recommend more frequent and comprehensive monitoring [30]. The potential use of a PICC line for the first 10-14 days post-infusion has been reported as aluable, allowing for frequent sampling at critical early timepoints (e.g., 1, 12, 24 hours) with less trauma to the patient. Monitoring should include platelets, renal function (cystatin C), markers of hemolysis (LDH, schistocytes), and complement activation (C3, C4, C5b-9). It was noted that tests for key complement components like soluble C5b-9 are often send-out labs, resulting in delays that hamper real-time clinical decision-making. It was also highlighted that some baseline abnormalities may complicate the interpretation. For example, patients with DMD may have pre-existing, spontaneous complement activation due to muscle tissue breakdown, complicating the interpretation of post-infusion changes.

3.5.1. A Case of unforeseen toxicity with C3 Inhibition in Danon disease. Findings from the Rocket Pharmaceuticals trial, presented by Jonathan Schwartz

A watershed landmark and tragic experience from the Rocket Pharma Danon disease trial has illustrated the severe end of the spectrum of innate mediated toxicity. To proactively prevent immune-complex-mediated TMA in the Danon trial involving high-dose AAV9, two patients were treated with a C3 complement inhibitor (pegcetacoplan) in addition to the standard immunosuppression regimen of rituximab, sirolimus and corticosteroids. Both patients developed profound hypovolemic shock and capillary leak syndrome manifesting at days 4-6 post-infusion. This was characterized by intravascular volume depletion, without cardiac failure or sepsis at time of initial presentation. One patient recovered; the other experienced refractory shock that required invasive hemodynamic support (extracorporeal membrane oxygenation, ECMO), with several complications including a large retroperitoneal hemorrhage and extensive refractory pulmonary opacifications. Life support was withdrawn approximately 3.5 weeks post gene transfer infusion in the context of extensive and non-resolving lung injury and subsequent sepsis. The leading hypothesis is that by inhibiting C3, the treatment blocked the C3-mediated opsonization of AAV particles by C3b, affecting clearance of AAV particles by the reticuloendothelial system (e.g., liver, spleen).

Without sufficient C3 levels, a much higher amount of AAV may then have entered endothelial cells, leading to a profound and fatal endovascular toxicity. The shock was preceded by a dramatic upregulation of cytokines associated with vascular permeability (IL-1 β , IL-8, MCP-1), suggesting a distinct inflammatory mechanism. The vector used in this phase of the trial had a much higher percentage of full capsids (74%) compared to an earlier phase (42%). While typically considered a marker of high quality, the hypothesis is that this led to a higher intracellular burden of TLR9-stimulating genetic material within cells, paradoxically contributing to the severe toxicity. Additionally, because of the underlying autophagic (LAMP2) deficit in Danon disease is also present in mononuclear and endothelial cells, there is potential for amplified intracellular innate immune activation specifically in this disorder (this hypothesis is currently under investigation). These observations and assessments led to the decision to reduce the vector dose for future patients (from 6.7×10^{13} GC/kg to 3.8×10^{13} GC/kg).

3.5.2. Case study in spinal muscular atrophy with progressive myoclonic epilepsy (SMA-PME): patient clinical course, treatment and retrospective analysis, presented by Ana Buj-Bello

Another case study of a severe early adverse event was presented by Ana Buj-Bello, along with the data obtained retrospectively. A female patient with an advanced stage of spinal muscular atrophy with progressive myoclonic epilepsy (due to mutations in the *ASAH1* gene) received an AAV9-based gene therapy on compassionate use.

The patient was stable for the first three days post-infusion. Subsequently, the patient developed a fever with an inflammatory syndrome and suspicion of complement activation. On day five, the patient experienced acute and refractory cardiovascular failure with cardiogenic shock, requiring ECMO and hemofiltration. The patient received Prednisolone 1 mg/kg on day 0, increased to 2 mg/kg on day 2 and 3mg/kg on day 5. Eculizumab was included on day 5, but she succumbed shortly after, on day eight.

Autopsy revealed no evidence of thrombotic microangiopathy or myocarditis. However, retrospective analysis of collected samples revealed an increase in some cytokines prior to vector injection, indicating a potential pre-existing inflammatory state. At the time of the AAV infusion, cytokine levels had returned to normal levels, likely due to preparatory suppressive therapy. However, the administration of the vector was followed by a rapid and significant increase in multiple cytokines. Further analysis of neutralizing antibodies was performed; as per protocol, the patient tested negative for neutralizing antibodies (NAb) prior to treatment. However, it was subsequently found that this child was positive for total binding anti-AAV9 IgG before treatment, with a titer of 1:157. Immediately post-infusion, total anti-AAV9 IgG levels increased before the expected rise in IgM.

A hypothesis was raised that the SMA-PME associated high levels of ceramides, which can be pro-inflammatory, may have played a role in the severe immune response. This outcome was potentially linked to both the presence of pre-existing binding anti-AAV IgG (despite being negative for neutralising antibodies) and the baseline, disease specific inflammatory state, underscoring the limitations of current patient screening criteria and the need for deeper individualized immunological investigation.

4. Liver-related adverse events

Chairs: Anil Dhawan and Rohit Kohli

4.1. The different mechanisms and timelines for the different types of liver involvement observed after AAV gene therapy

Liver toxicity is a primary concern in the administration of AAV-based gene therapies [31]. The liver, often an unintended bystander organ in therapies targeting muscle or the nervous system, receives a substantial portion of the vector dose, leading to significant adverse events. A PubMed search for "AAV liver and toxicity" reveals a significant increase in publications over the past three decades, indicating growing recognition and investigation of this emerging toxicity. Across various clinical protocols encompassing approximately 220 patients, the rate of serious adverse events classified as acute liver injury is about 5.5%.

A critical distinction is made between acute liver injury and liver failure. Acute liver injury is typically defined by a rise in liver enzymes (e.g., ALT, AST, GGT) above a two- or three-fold threshold. This can be transient or long-term but may progress (~10%) to liver failure and a need for a lifesaving liver transplantation. Liver failure is of course a more severe condition characterized by synthetic dysfunction, primarily indicated by a rise in prothrombin time (INR > 2.0), unresponsive to vitamin K. This represents a desperate management situation and necessitates expert care in or in consultation with a liver center.

The pathophysiology of AAV-induced liver damage is complex, involving both the vector components and dose and the host's immune system, often influenced by underlying disease states. A consensus timeline suggests two primary phases of immune-mediated liver toxicity: (1) Early innate response (first wave), which occurs shortly after vector administration and reflects a complex acute immune reaction to AAV gene transfer (as discussed under TMA) and the AAV capsid. (2) Delayed adaptive response (second wave), typically emerges around 8-9 weeks post-dosing. This is thought to be an adaptive immune response, involving T-cell directed against the capsid proteins [3]. This

delayed response has been particularly noted in SMA patients, leading the U.S. Food and Drug Administration (FDA) to issue onasemnogene abeparvovec (Zolgensma) a black-box warning for acute serious liver injury [32,33], but more recently also in DMD and in limb girdle muscular dystrophy. Delayed immune response against other components of the viral vector are also likely to be implicated.

Liver biopsies from affected patients reveal a range of pathologies, including various forms of inflammation, presence of CD8+ positive cells, hepatocyte dropout and steatosis [34]. In fatal DMD cases, findings included marked ductular reaction and cholestasis, with a neutrophilic predominance despite the presumed T-cell mediated mechanism [35]. Parallels to organ rejection, the cellular response observed shares similarities with acute cellular rejection in liver transplantation, which involves lymphocytic infiltration and organ damage. AAV can induce autophagy in hepatocytes, a process required for effective transduction. Drugs used in immunosuppression regimens can modulate autophagy, thereby changing the kinetics and efficacy of vector processing and potentially influencing toxicity.

The patient's baseline condition is a critical variable in determining risk and severity. In MTM1 the disease itself involves defects in intracellular and canalicular transport of bile acids within hepatocytes, affecting the canalicular membrane system. This creates an underlying liver pathology that makes patients more susceptible to AAV-induced hepatobiliary toxicity. In SMA older patients with obesity have shown impaired fatty acid oxidation in the liver, which may predispose them to elevated liver enzymes and hepatic steatosis [36,37]. Patients with DMD are often in a profoundly inflammatory state before treatment, which may exacerbate the innate immune response to the vector but also complicates management [8,38]. Long-term steroid use can also lead to obesity and hepatic steatosis [39].

Pre-existing or latent viral infections (e.g., hepatitis B, CMV, EBV, adenovirus) can significantly alter the intracellular environment of hepatocytes. For example, the HBx protein from hepatitis B can accelerate AAV transport to the nucleus, potentially increasing transduction efficiency and vector load in hepatocytes. Reactivation of these viruses under immunosuppression is a major concern, reminiscent of post-transplant lymphoproliferative disease (PTLD). A previous, even resolved, infection can prime the liver's innate immune memory, potentially altering its reaction to a subsequent AAV challenge.

4.2. Insights from specific disease programs and case studies

4.2.1. Myotubular Myopathy (MTM1), presented by Amitava (Tav) Ganguli for Astellas

Hepatobiliary disease is now more widely recognized as being a feature of the multisystemic involvement of XLMTM1 natural history, and a potential critical factor to treatment related liver toxicities. The initial MTM1 trial (Aspiro/Audentes Therapeutics) revealed the disease's inherent liver pathology, which predisposed patients to severe hepatobiliary toxicity, leading to tragic deaths of four patients with severe cholestatic liver dysfunction [40,41]. Until recently, the prevalence and severity of the liver involvement in MTM1 was not well understood; this aspect of the disease is also not recapitulated under normal conditions in the MTM1 deficient mouse model of the disease.

Astellas' new program has been redesigned based on these learnings: (1) Uses an engineered capsid (MyoAAV 3.8) with higher muscle tropism. (2) Drastically reduced dose (1×10^{12} vg/kg), which is several hundred times lower than the previous trial. (3) Higher percentage of full capsids (70-80% vs. 40%). (4) Stricter liver inclusion criteria. (5) A robust prophylactic regimen of IV/oral prednisone and sirolimus starting pre-dosing and continuing for about 20 weeks. It was noted that, given the liver's involvement in the underlying disease pathology [42], therapeutic strategies should ideally also aim to target the liver. A product that allows at least partial expression in the liver may be desirable to ensure more comprehensive disease modification and avoid toxicities.

4.2.2. Duchenne Muscular Dystrophy (DMD), presented by James Richardson for Sarepta

Two DMD fatalities occurred in older (15–16 years old) non-ambulatory boys, after approximately 800, predominantly younger patients, had been dosed without similar events. The gene therapy administered is dosed at a recommended dose of 1.33×10^{14} vg/kg capped at 70 kg, the first case received a total dose of 9.31×10^{15} vg, the maximum total dose per the commercial prescribing information, and the second received 7.18×10^{15} total vg. One patient was identified to be heterozygous for alpha-1 antitrypsin deficiency.

The cases were notable for a prodrome (5 weeks in the first case and 4 weeks in the second case) of being clinically stable despite abnormal LFTs, thrombocytopenia, progressive hyperbilirubinemia seemingly out of proportion to other markers followed by a sudden, rapid clinical deterioration. The first patient was found acutely neurologically obtunded on the ward and his subsequent demise was attributed to intracranial haemorrhage secondary to the synthetic liver failure, at autopsy his liver showed extensive ductular transformation and periportal collapse, steatosis, cholestasis, and jaundice, with minimal inflammation and fibrosis. The second patient developed a systemic hyperinflammatory syndrome resembling macrophage activation syndrome or secondary hemophagocytic lymphohistocytosis (HLH) with more marked thrombocytopenia, extremely high GGT levels, hyperbilirubinemia and markers of cholestasis, which again seemed out of proportion to other markers. The demise of the second case was attributed more to an uncontrollable systemic inflammatory response rather than primary liver synthetic failure, though the liver was clearly involved with a liver biopsy showing features consistent with immune mediated liver injury. More details of these cases have been separately published [35]. Of note, a third fatality occurred in a limb-girdle muscular dystrophy patient using the same platform with a different manufacturing process and dose.

4.2.3. Spinal muscular atrophy (SMA), presented by Hugh McMillan and Francesco Muntoni

The SMART study of older, heavier SMA patients found that over 80% experienced subclinical elevation of serum transaminases. A third (9/24; 38%) required doses of > 2 mg/kg/day of corticosteroids, and 25% still required corticosteroids after one year due to "grumbling" mild persistent transaminitis [43]. Chronic inflammation raises theoretical concerns regarding long term risk of fibrosis or carcinoma, and increasing attention is being directed to the use of alternative forms of immunomodulation (e.g. sirolimus) in selected cases. Liver biopsies from four children who developed subacute liver failure following gene replacement therapy for SMA showed hepatocellular injury and inflammation, with reported findings including hepatocyte ballooning, acute hepatocellular necrosis, parenchymal inflammation with abundant CD8+ T-cell lymphocytes, and fibrosis [33,34]. Of note, it is also conceivable that SMN deficiency, together with the known impairment of fatty acid oxidation in patients with SMA [44], may predispose this population to a higher likelihood of liver-related toxicities.

In a case reported by Francesco Muntoni, a single SMA patient experienced a severe response to onasemnogene ABEPRVVEC (Zolgensma) and provided critical insights into toxicities associated with impurities and flawed vector integrity. The patient developed early liver failure, which was medically managed. Upon liver biopsy, plasmid contamination was found. A probe for human SMN confirmed transduction of 29% of liver cells. However, probes complementary to plasmid sequences used in manufacturing detected the presence of plasmids that were not part of the therapeutic vector genome. All three plasmids used in manufacturing were found to be present as contaminants. Furthermore, long-read sequencing revealed high levels of vector genome concatemerization and rearrangements. Some concatemeric structures were found to be larger than the maximum packaging capacity of the AAV capsid, suggesting that recombination events may have occurred *in vivo* after transduction. These findings raise concerns

that plasmid contaminants could facilitate the amplification of vector components within the cell and possibly contribute to observed toxicities [27]. However, there is no analytical information available regarding any of the other products in clinical use, so it is not possible to compare this finding to any of the remaining vectors in use.

4.3. Immunosuppressive regimens to consider. Reactive versus prophylactic therapies

Effective management to mitigate liver toxicity relies on a comprehensive strategy encompassing pre-treatment screening, diligent monitoring, and tailored immunosuppression. The current challenges encompass lack of chronic toxicity data in animals studies, different immunosuppression protocols in different studies, minimal liver biopsy data, lack of systematic data collection for post marketing adverse incidents, and deficiency of unified protocols for the management of liver toxicity in the context of AAV gene replacement therapies [45].

4.3.1. Patient management: a three-pillar approach

Pillar 1: Pre-treatment screening and risk stratification: A standardized minimum dataset for screening is proposed to identify and stratify at-risk patients before AAV administration.

A basic set of liver function tests is essential to rule out unrelated liver conditions. This would include screening for herpes viruses (CMV, EBV) and other hepatotropic viruses. Knowing a patient's exposure status allows for proactive monitoring and management, as immunosuppression can trigger reactivation and change the kinetics of transduction. Screening for common metabolic liver diseases such as alpha-1 antitrypsin deficiency is considered important as these can increase risk. It was also suggested that the patient's baseline inflammatory milieu could be a predictive factor for their response to the vector. The discussion highlighted that this is for risk stratification and tailored monitoring, and not necessarily exclusion from treatment.

Pillar 2: Monitoring protocols. There is a call to harmonize monitoring protocols across different trials and therapies. Regular monitoring of ALT and AST is standard. Given that cholestatic injury is associated with poorer outcomes in drug-induced liver injury, adding GGT and bile acids to monitoring panels is strongly recommended as a sensitive marker for this type of damage. In DMD, where muscle breakdown elevates baseline CK, the ALT/CK ratio was proposed as a more sensitive signal of emerging liver injury than ALT alone, as a drop in CK concurrent with stable or rising ALT can unmask a problem [46]. Monitoring should be frequent, especially in the first few months post-dosing, to catch the two waves of immune response.

Pillar 3: Immunosuppression regimens. Immunosuppression strategies are evolving rapidly, drawing heavily on decades of experience from solid organ transplantation, where agents such as azathioprine, glucocorticoids, ciclosporin and tacrolimus, mycophenolate, sirolimus and everolimus as well as antibodies are used routinely to mitigate immune related toxicities. Two approaches were discussed - prophylactic vs. reactive:

Reactive: The older approach, where immunosuppression (typically corticosteroids) is initiated only after liver enzymes rise. Corticosteroids are often used as first line with tacrolimus or sirolimus for more severe or refractory cases. Use of additional agents is emerging (IVIg, rituximab, anti-complement antibodies).

Prophylactic (induction): The increasingly favored approach, where immunosuppression is started before or at the time of vector administration to pre-emptively blunt the immune response. This front-loading is considered more effective than trying to reverse an established immune reaction. It was however noted that prophylactic immunosuppression can be considered "investigational" by some regulatory authorities (e.g., in Canada and China), creating operational hurdles for global clinical trials.

4.3.2. Drug classes and strategies

Corticosteroids: Remain the mainstay for both prophylactic and reactive treatment. However, they have significant side effects (catabolism, weight gain, bone health), especially in catabolic or steroid-naïve patients. It was noted that while all protocols include steroids as part of immune mitigation strategy, administration time varies from study to study. Administering steroids for a period (e.g., 3-7 days) before gene therapy was discussed to blunt the initial innate immune response. Other therapies such as sirolimus also require several days up to a week to achieve therapeutic drug levels, with age specific differences in its metabolism. The significance of early use of steroid sparing agents was highlighted.

Calcineurin Inhibitors (e.g., tacrolimus) & mTOR inhibitors (e.g., sirolimus): Increasingly used as part of prophylactic maintenance regimens. Sirolimus is noted for potential side effects like leg pain and mouth ulcers in young children but may offer some tolerance benefits.

Antibodies: Used as "bigger guns" for severe or refractory cases. Basiliximab (an IL-2 receptor antagonist) is used in transplant as a steroid-sparing induction agent and is proposed for consideration in gene therapy.

4.4. The Role and controversy of liver biopsy

The necessity and timing of liver biopsies were a major topic of discussion, highlighting a conflict between clinical need and practical implementation. There is often a disconnect between biochemical markers (liver enzymes) and the actual histological state of the liver. A patient can have normal enzymes but active inflammation on biopsy. Liver biopsy is considered the definitive "gold standard" to understand the mechanism of injury, confirm the need for escalating therapy, and provide a clear rationale to families for using more toxic drugs. Biopsies are not recommended for all patients. Instead, they are proposed for specific clinical scenarios, primarily when a patient's liver enzymes are not settling despite standard therapy and an escalation of immunosuppression is being considered.

Specific barriers and concerns were discussed. (1) There is significant "biopsy fear," and obtaining consent can be difficult, especially for a procedure framed as a research question rather than a direct benefit to the child. (2) Insurer acceptance and hospital capability are potential hurdles. (3) While the complication rate is very low (<0.1%) at experienced centers, it is still an invasive procedure.

A strong case was made that if biopsies are performed, they should be leveraged for maximum scientific value beyond standard histology. This includes applying advanced techniques like single-nuclear RNA sequencing and proteomics to create a detailed map of cellular responses at baseline and post-treatment. This was proposed to be conducted in a select, high-risk cohort as part of a formal research protocol to advance the entire field. It was also suggested that consent for potential future biopsies could be included as an optional item in the main trial consent, with a separate, specific consent form provided only if an event occurs, reducing the upfront burden on families.

Furthermore, there is a need for a panel of expert pathologists (e.g., cardiac, muscle, liver) to review existing biopsy samples to ensure that all relevant questions, such as distinguishing myocarditis from underlying pathology, have been thoroughly addressed.

Ultimately, the long-term solution likely lies in vector innovation, specifically the development of AAV capsids with reduced liver tropism. In the interim, there is an urgent need for collaborative efforts to standardize patient management, develop better predictive biomarkers, and deepen the mechanistic understanding of liver injury to improve the safety profile of these therapies.

Parallel to long-term research planning, the discussion emphasized practical steps that could be implemented immediately with minimal funding. An immediate action point is to define and agree upon a standardized, minimum set of clinical and laboratory parameters to be collected systematically on all patients, which will be a future

publication of the group. Discussed parameters include pre-screening for viruses such as CMV and herpes, as well as collecting baseline levels for markers like bile acids and fibrinogen. Another discussion point was systematic assessment of pre-existing and concomitant host and disease related conditions, such as assessing the baseline inflammatory status of patients, particularly older individuals. This could involve using non-invasive techniques like elastography to assess for fatty liver disease, even outside the context of a clinical trial.

5. Cardiac adverse events in AAV gene therapies

Chairs: Barry Byrne, Karim Wahbi and Qi Shen

5.1. Findings from post-mortem cases, considerations for patient selection and management

The discussions identified at least three distinct types of cardiotoxicities, each with different underlying biology, clinical presentation, and timeline. A critical theme is the need for precise terminology, distinguishing early "myocardial inflammation" from later "myocarditis" to guide appropriate management.

Preclinical studies reveal that direct toxicity from overexpression of the therapeutic transgene in cardiomyocytes is a significant risk. This phenomenon is highly transgene-dependent, with different proteins inducing toxicity at varying dose levels and through distinct cellular pathways. The following specific preclinical examples were presented by Isabelle Richard: gamma-sarcoglycan (γ -SG), LGMD-R5: Accumulation of this protein in cardiomyocytes leads to an increase in autophagy and activation of cell death pathways; FKRP, LGMD-R9: Protein accumulation causes cellular stress, a blockade of autophagy, inhibition of the proteasome, and activation of cell death pathways. Lethality was observed in preclinical studies at doses of 7.5×10^{13} vg/kg; Calpain 3 (CAPN3), LGMD-R1: As a protease, unregulated expression of CAPN3 leads to the destruction of various proteins, causing lethality at very low doses (6×10^{12} vg/kg); Micro-dystrophin: Overexpression leads to the invasion of the intercalated disc, modifying channel functions and potentially causing electrical abnormalities, fibrosis, and cell death. Lethality was noted at a dose of 1×10^{14} vg/kg [47–50].

The key point is that the heart appears more sensitive to overexpression toxicity than skeletal muscle. A potential solution demonstrated preclinically is the insertion of a heart-specific microRNA target sequence into the vector's expression cassette (miR208a). This "de-targets" the heart, drastically reducing transgene expression in cardiomyocytes and preventing toxicity without impacting expression in skeletal muscle.

Acute immune-mediated myocardial inflammation represents an early, severe event occurring approximately 5 to 10 days post-AAV infusion. It is not a classic myocarditis involving cellular infiltrates but is instead driven by the innate immune system and complement activation [19]. This type of event starts at day 5 and peaks between day 9 and 10. It is triggered by the formation of AAV vector-antibody complexes, which activate the classical complement pathway. AAV capsids (notably AAV9) can also directly activate the alternative pathway, creating an amplification loop that drives a dysregulated response.

The critical mediators of tissue damage are the anaphylatoxins C3a and C5a, which cause endothelial injury, capillary leak, and myocardial inflammation. This leads to myocardial edema, increased myocardial stiffness, and acute cardiac dysfunction. Patients can experience rapid decompensation with pulmonary edema and respiratory distress. Autopsy and biopsy specimens from these acute events are notable for the absence of lymphocyte infiltrates, confirming a non-cellular mechanism [51]. This contrasts fundamentally with later anti-transgene mediated myocarditis. Different AAV capsids appear to have different propensities for activating complement. Data suggests AAV9 and AAVrh74 behave differently in this regard.

Late-phase anti-transgene myocarditis is a more classically

understood immune event that occurs later in the post-treatment course. It is characterized by an adaptive immune response, where T-cells recognize the newly expressed transgene protein as foreign. Unlike the acute inflammatory events, biopsies from these cases show significant mononuclear cell and lymphocyte infiltrates. This form of myocarditis may be responsive to glucocorticoids and other immunosuppressants, whereas the early innate responses are not.

5.2. Considerations on the selection of patients with cardiac involvement, monitoring and diagnosis

Selecting appropriate candidates for gene therapy is a major challenge, requiring a balance between safety and efficacy, especially for patients with pre-existing cardiac involvement.

Patients with underlying cardiomyopathy or heart failure are more susceptible to adverse events. Their compromised cardiac function provides less physiological reserve to withstand insults like fluid retention from corticosteroids or direct cardiotoxic effects from the therapy. The natural history of the specific disease is also critical; for example, patients with laminopathies are at high baseline risk for sudden death from arrhythmias, which can be difficult to distinguish from a treatment-related event.

A key challenge is defining appropriate exclusion criteria. A common sentiment is the difficulty in relying on a single "magical number," as patient risk is multifactorial.

The following criteria (Table 2) have been commonly used to exclude patients at higher risk of cardiac events.

Harmonization of selection and exclusion criteria was identified as a priority. Current criteria vary and include items specific to disease pathology and to the particular gene therapy platform used. Beyond theoretical frameworks, these criteria should be empirically validated using both existing patient datasets and prospective data from future treated cohorts to ensure they reliably predict safety and efficacy across populations.

To move beyond simple LVEF, the workshop participants advocated for a more comprehensive assessment to encompass collection of findings, including LVEF, mitral flow propagation wave (indicates stiffness), and MRI-derived strain, rather than a single parameter. It was highlighted that cardiac MRI is considered more reliable than echocardiography, and it is essential for establishing a pre-treatment baseline if this can be performed without general anaesthetic. It allows for the assessment of fibrosis (LGE) and, critically, circumferential strain, which is viewed as a highly predictive measure of future dysfunction, particularly in DMD.

Effective post-dosing monitoring is essential for early detection and

intervention. The discussion highlighted the utility and limitations of various tools.

Troponin: High-sensitivity troponin is now widely used but has become a "nuisance" biomarker. Elevations are frequently observed, even in asymptomatic patients (e.g., in SMA trials) or as part of the natural history of the disease (e.g., DMD), without correlating with cardiac dysfunction. This creates significant "noise" and anxiety to patients.

Natriuretic Peptides (BNP/NT-proBNP): These markers may be more clinically relevant, as dramatic increases have been observed at the point of cardiac decompensation, suggesting they are a better indicator of functional decline.

CBC - platelets: early decline in platelet counts even before overt thrombocytopenia, may precede complement activation and is a good indicator of subclinical endothelial injury.

Complement components: Monitoring C3 and C4 levels can herald the onset of acute complement-mediated inflammation, as their consumption mirrors the increase in the terminal complement complex (C5b-9). However, causality should be interpreted with caution, as tissue injury itself may drive complement activation.

Hemoglobin: An increase in hemoglobin (hemoconcentration) was highlighted as an early and readily available sign of the capillary leak syndrome associated with severe, acute inflammation.

Arrhythmia Detection: There is an ongoing debate about the increased use of long-term arrhythmia monitoring in the forms of extended Holter monitors (e.g., 2-week) and implantable loop recorders that are being used more frequently. In diseases with a known high risk of sudden cardiac death, arrhythmia monitoring provides critical prognostic information. However, in low-risk conditions, they may generate artifacts and increase patient anxiety. There is also a lack of consensus on how to interpret the data, particularly in a clinical trial context where the local site may not routinely use such devices.

5.3. Management of cardiac toxicity

The management approach must be tailored to the specific type of cardiotoxicity. In managing acute myocardial inflammation, experience from severe cases underscores a clear management path for the early, innate-driven events. Glucocorticoids alone may not be effective for these early innate immune responses. Management hinges on aggressive supportive care in a highly specialized ICU setting with cardiovascular expertise. This includes hemodynamic support to maintain adequate preload in a stiff, preload-dependent heart. In extreme cases, therapeutic plasma exchange has been used to successfully reverse the condition. However, this comes at the significant cost of also removing the therapeutic vector from circulation.

A case of acute immune-mediated myocardial inflammation in the Pfizer Ph1b trial was presented by Dan Levy: The ultimately fatal course in a 16-year-old DMD patient highlighted the rapid progression of acute toxicity. The patient developed intractable nausea and vomiting around day 4-5, followed by a troponin rise, a profound drop in ejection fraction, and evidence of capillary leak (hemoconcentration). He decompensated and expired on the same day he was hospitalized. At the time, monitoring protocols were suboptimal, and the event was not fully understood. Following the fatal case, Pfizer noted that troponin fluctuations occurred in both gene therapy-treated and placebo arms, underscoring background variability. In addition, one of the patients in the Phase 3 clinical trial who developed thrombotic microangiopathy (TMA) also had an elevated troponin-I and pro-BNP during the first week after infusion with the AAV9-based-gene therapy, fordadistrogene movaparvovec. This patient had no cardiac symptoms or new cardiac imaging abnormalities; he did not receive steroids, and the elevated troponin levels declined and fully normalized ~2 months post dosing.

Two cases of late-phase anti-transgene myocarditis in the Pfizer trial were presented by Qi Shen: Supra-high troponin levels, particularly when accompanied by supportive imaging findings, were considered

Table 2
criteria commonly used to exclude patients at higher risk of cardiac events.

Criteria	Specific metric / condition	Common thresholds / notes
Ventricular Function	Left ventricular ejection fraction (LVEF)	<40% or <50% is a common exclusion. A patient with LVEF <40% has a median of ~2.5 years until cardiac decompensation in DMD.
Cardiac History Arrhythmias	History of heart failure / decompensation Uncontrolled ventricular arrhythmias	Patients with a prior event are at extremely high risk for another. In high-risk diseases (e.g., laminopathies), a pre-implanted ICD has often been mandatory for trial inclusion.
Fibrosis	Myocardial Fibrosis / LGE on MRI	The presence of significant fibrosis indicates a higher risk, but a specific quantitative threshold for exclusion remains a topic of debate and lacks consensus.
General Health	Significant cardiovascular comorbidity	History of stroke, myocardial infarction, coronary artery disease.

suggestive of myocarditis. In their experience, two patients with markedly elevated troponin and imaging abnormalities met criteria for myocarditis; both were treated with corticosteroids and subsequently returned to baseline.

6. AAV gene therapy emerging toxicities

Chairs: Juliette Hordeaux, Carsten Bönemann

New and emerging toxicities associated with high-dose adeno-associated virus (AAV) gene therapy were presented and analysed. Data from non-human primates (NHPs), clinical trials, and a fatal DMD case identify two emerging risk domains: (1) early endothelial cell injury driven by rapid, widespread transgene expression, and (2) fatal recurrent rhabdomyolysis in a DMD patient with a Becker-like genotype, highlighting a novel and poorly understood risk profile with potential genetic susceptibilities and atypical immune responses.

6.1. Acute systemic toxicities: endothelial injury as a unifying mechanism

Key findings indicate that severe adverse events in NHPs, including profound liver injury and capillary leak syndrome, are correlated with extremely high and widespread transgene expression in all liver cell types as early as Day 3 post-infusion. This suggests a direct, dose-dependent effect that may bypass the rate-limiting step of second-strand DNA synthesis. The proposed common denominator for these early events—including TMA, capillary leak syndrome, and liver injury—is endothelial injury. Critical clinical insights include the identification of early warning signs, such as rapid body weight gain from fluid retention in NHP, and the necessity of monitoring blood markers, especially decrease in platelet levels, as early as Day 3 to capture the initial onset of toxicity.

6.1.1. Evidence from non-human primate (NHP) and human data

A comparative analysis of clinical data from the onasemnogene abeparvovec (Zolgensma) STRIVE trial (22 patients) and a meta-analysis of NHP studies (36 primates receiving high-dose AAV) revealed critical insights into the timing and nature of acute toxicities [52].

The meta-analysis of NHP studies demonstrated a dose-dependent effect for severe adverse events. At doses equal to or higher than 1×10^{14} vg/kg, the rate of these events was as high as 30%, presenting as transaminitis (elevated ALT and AST enzymes), thrombocytopenia (platelet drop), body weight gain (due to oedema and fluid in cavities) and non-inflammatory acute liver injury within days post dosing [53].

Early onset of toxicity observed in NHP data showed liver enzyme (ALT) elevations and platelet drops on Day 3, an earlier timepoint than previously captured in clinical protocols that usually start monitoring at Day 7. In NHPs, ALT spikes at Day 3 and begins to resolve by Day 7, mirroring the Day 7 peak seen in the human data. Liver injury was characterized by necrosis, endothelial cell injury, stellate cell activation and fibrosis, observed as early as five days post-infusion. This is a non-inflammatory pathology, distinct from later, immune-mediated liver damage. The timeline suggests that crucial initiating events occur within the first 72 hours, encouraging the inclusion of earlier monitoring in toxicology studies as well as clinical trials [53].

A biphasic cardiac toxicity was observed in one study. In the early phase (Days 3-7), two animals developed a severe endothelial injury syndrome with capillary leakage, requiring early euthanasia. There was no evidence of myocarditis; histology showed no inflammatory infiltrates. A key predictive clinical sign was a rapid body weight gain of 10-20% within a day, resulting from oedema and fluid accumulation. This suggests that non-invasive monitoring like daily weight checks could be a valuable early warning system. In the late phase (Day 60), an animal scheduled for necropsy presented a second spike in troponin levels, with no clinical symptoms. Histopathology revealed myocarditis with CD8+ T-cell inflammatory infiltrates. This was determined an

immune dependent myocarditis in response to an epitope in the human transgene non-homologous to the endogenous NHP protein [52].

6.1.2. Molecular Insights from RNA seq: rapid and widespread transgene expression

Liver single-nuclear RNA sequencing (snRNA-seq) results in NHP study were presented. NHPs received a high dose with either reporter and non-reporter transgene delivered by clade F AAV capsids (GFP delivered in AAV-PHP.eB and PHP.B, and therapeutic transgene hu68.tg3 delivered in PHP.eB and AAV9) [53]. NHPs euthanized at Day 3-5 due to severe toxicity, presented transgene expression in all liver cell types, including hepatocytes, endothelial cells, Kupffer cells, and others. In contrast, animals with no SAEs showed only ~15% of liver cells positive for the transgene, with expression confined mostly to hepatocytes at the two-week time point (with the same ubiquitous promoter). This suggests that severe acute toxicity is associated with rapid and promiscuous transgene expression in the liver of particular intensity in animals who develop this complication.

Results from human RNAseq [54] from post-mortem samples of a DMD patient, dosed with AAV9 (CK8 promoter) who succumbed to acute respiratory distress syndrome (ARDS), revealed the highest levels of transgene transcripts in the lung, with 20% of endothelial cells expressing the transgene transcript and less than 2% of cells in the liver and heart expressing transcripts. While protein expression was observed, this finding spatially correlates the site of highest transgene expression (lung alveolar capillaries) with the organ of fatal injury (ARDS).

It was noted that NHPs are considered a valuable model for linking mechanistic hypotheses to clinical outcomes. However, a major limitation is that only about 30% of NHPs develop the severe adverse event profile of interest. This makes prospective studies designed to test interventions or compare vector manufacturing processes prohibitively expensive and often inconclusive, as demonstrated by previous experiments where no animals developed SAEs.

6.1.3. Discussion: A multi-hit model of endothelial injury

The evidence suggests that endothelial injury is a common denominator in early AAV toxicities and an emerging pathology that should be monitored. A proposed "multi-hit endothelial injury" model was discussed with the following combination of factors: Hit 1: Direct AAV effect by rapid, high transcription of AAV genomes in endothelial cells causing cellular stress and injury. The temporal and spatial correlation between high transcript levels and organ damage is a key piece of evidence. Hit 2: Complement-mediated injury via activation of the fluid-phase complement system, a known mechanism in thrombotic microangiopathy (TMA), contributing to endothelial damage. Hit 3: Pre-existing condition of endothelial cells affected by the underlying disease's inflammatory state may determine the severity of the response to AAV administration.

7. Vector and host factors contributing to AAV gene therapy toxicities

Chairs: Jan Kirschner and Hildegard Büning

Clinical data and recognized examples of host susceptibilities contributing to gene therapy-associated toxicities were discussed. A recurring theme was that adverse events are multifactorial, arising from a combination of vector dose, promoter strength, transgene expression, vector quality, and pre-existing patient vulnerabilities. Clinical evidence further suggests a direct correlation between higher vector doses and stronger promoters and the incidence of treatment-related toxicities

7.1. Dorsal root ganglion (DRG) toxicity and CSF pleocytosis in intrathecal AAV administration

Carsten Bönemann presented two examples involving intrathecal AAV9-mediated gene replacement therapies that employed different

promoters and dosing: the giant axonal neuropathy (GAN) trial [55] and a compassionate-use case for hereditary spastic paraplegia type 50 (SPG50).

In the GAN trial, the investigational product utilized a ubiquitous and intentionally relatively weak JeT promoter at a maximum dose of 3.5×10^{14} vg/patient. At three months post dosing there was a dose dependent cerebrospinal fluid (CSF) pleiocytosis, predominantly consisting of B cells [55]. This inflammatory response within the CSF compartment is thought to represent a reaction to the AAV capsid, although the precise mechanism remains under investigation. The pleiocytosis was responsive to corticosteroid treatment, leading to extension of steroid coverage beyond the three-month point, followed by a taper, and normocytosis in the CSF at the 12-month point. Importantly, no meningeal enhancement was observed on MRI, and no corresponding clinical symptoms were identified. Long-term follow-up further demonstrated re-emergence of sensory nerve action potentials (SNAPs) as a signal for treatment efficacy and DRG restoration rather than toxicity.

In contrast, a case of DRG toxicity was observed in the SPG50 compassionate-use protocol, in which a 13-year-old boy received intrathecal administration of the same AAV9 capsid with a stronger JeT promoter at a total higher dose of 1×10^{15} vg [56]. Clinical findings included elevated CSF protein levels without pleiocytosis, decreased distal sensation, as well as reduced sensory nerve conduction amplitudes relative to baseline. Two SMA participants (2.7%) in the intrathecal onasemnogene abeparvovec (OAV101 IT) arm of the Phase 3 STEER study (NCT05089656) reported signs and symptoms potentially indicative of DRG toxicity prompting the use of multiple symptomatic and immunosuppressive treatments [57]. Notably, DRG toxicity does not appear to be restricted to protein-expressing transgenes. A separate trial aimed at SOD1 suppression, using a protein-free microRNA transgene, also resulted in DRG toxicity [58], suggesting that the RNA molecule itself may contribute to toxicity.

7.2. Advancements in vector design and manufacturing

Engineered capsids and hence lower doses required could contribute to overcoming immune reactions against AAV capsids. The current Phase 1 trial for MTM1 (NCT07052929) by Astellas utilizes an engineered muscle-tropic capsid (MyoAAV3.8) at a systemic dose approximately 100 times lower than in previous studies (1×10^{12} vg/kg), aiming to improve the safety profile by concentrating expression in the target tissue.

Furthermore, there is a need to continue to improve vector quality and manufacturing processes, although there have been some advances leading to improved critical quality attributes. The new MTM1 gene therapy product, for example, has a full-to-empty capsid ratio of 70–80%, a significant improvement over the ~40% in the previous product. Nevertheless, techniques to assess aggregation and post translational modifications need further development and implementation.

7.3. A Call for collaborative, consortium-based research

It was discussed that genetic susceptibility could be assessed by genetic whole association studies on single nucleotide polymorphisms (SNPs). There is a strong consensus on the need to create a collaborative consortium including sponsors, academicians, physicians, and registries to perform WGS on treated patients, so data can be stored and analysed. As exposed patients continue to increase, this data would allow identification of genetic predictors (e.g., SNPs in immune-related pathways) that predispose certain individuals to severe adverse events.

A proposal for data and sample sharing was made to create a centralized platform for sharing information. This could include immunomodulatory protocols, inventories of available biosamples, and key analytical assays, creating a shared resource to accelerate collective learning across all programs. The community should work toward a

consensus on standardized clinical screening protocols (e.g., for assessing liver steatosis) to ensure that data generated across different trials are comparable and can be collated for further analysis.

8. Bridging preclinical and clinical gaps in gene therapy safety

Chairs: Francesco Muntoni and Barry Byrne

Discussion focused on additional preclinical work and assay developments that are needed to better predict and model toxicities, and to inform clinical preventive and reactive immunosuppression regimens to mitigate SAEs. The central challenge is the current inability to reliably stratify patients based on their risk of developing complications such as thrombotic microangiopathy (TMA), acute liver injury, and other immune-mediated toxicities. The discussion outlined a dual-pronged strategy: implementing immediate, practical data collection standards that can be adopted in the short term, alongside developing more complex, long-term research initiatives that require funding and collaboration.

Key proposed actions include leveraging a fresh peripheral blood mononuclear cell (PBMC) assay for pre-administration susceptibility screening, initiating a pilot program for whole-genome sequencing (WGS) to identify genetic risk factors, and conducting deep retrospective analyses of existing clinical trial data and banked patient samples. The foundational theme is the critical need for a collaborative framework, potentially dedicated working groups or consortium to secure funding, standardize protocols (SOPs), and navigate the logistical and ethical hurdles of multi-center data and sample sharing.

8.1. The challenges of predicting and mitigating severe adverse events (SAEs)

There is a recognized lack of reliable biomarkers for critical complications, including endothelial injury syndrome development. The overarching goal is to move towards effective risk stratification to improve patient safety.

A significant concern is the pre-existing pro-inflammatory state of certain patient populations, such as older DMD individuals who have undergone long-term steroid treatment and may have developed conditions like fatty liver. This underlying inflammation is hypothesized to lower the threshold for an adverse reaction, fitting a "multi-hit" model where the gene therapy acts as a second hit on a sensitized system.

A key question was raised about the assumption that different severities of an adverse event, such as acute liver injury (ALI) versus acute liver failure, share the same underlying pathogenesis. It was proposed to analyze data by separating adverse events into "waves," such as an early innate immunity-driven phase and a later T-cell-mediated phase, to better understand causality and triggers.

8.2. Proposed research strategies and methodologies

A variety of specific assays and large-scale research strategies were proposed to generate predictive insights. These range from *in-vitro* functional assays to broad genomic and proteomic screening.

8.2.1. Pre-administration susceptibility screening

A peripheral blood mononuclear cell (PBMC) assay was presented by Bradley Hamilton (patent: WO2024226496) as a tool for assessing patients' immune susceptibility prior to gene therapy infusion. This assay involves stimulating fresh PBMCs isolated from patients with a vector and measuring the resulting immune response in the supernatant. The assay measures cytokine release (cytokine release of ~80 different cytokines), in response to AAV and can also test individual responsiveness to various immunosuppressive agents. Critically, the supernatant containing the response markers can be frozen for later analysis, making a distributed collection model feasible where local sites perform the initial cell culture. The assay measures key inflammatory markers implicated

in TMA, including IL-1 β , MCP-1, and Interleukin-8. While ideally performed days before infusion to inform point-of-care decisions, the current workflow in clinics often means the blood is drawn on the day of infusion, with results available by day two.

8.2.2. Genetic susceptibility (whole genome sequencing)

The proposal to use whole-genome sequencing (WGS) to identify genetic variants that predispose patients to SAEs was a recurring theme.

A concrete proposal was made to launch a pilot study to establish a workflow. The goal is to collect and sequence a small number of samples, with potential support from advocacy organizations. A concern was the challenge obtaining consent for WGS presents, as patients' families may already be burdened with multiple consent forms. A streamlined, expedited protocol for de-identified samples was suggested as a path forward. The viability of using spit tests versus whole blood for high-coverage WGS was discussed as a key logistical question, as spit tests would simplify collection from international sites, however blood would be preferable. A critical need was identified for a sponsor-independent, open-source framework to host and share the genomic data to instill trust and encourage broader participation. The group will continue to work on this proposal.

8.2.3. Retrospective analysis of clinical data and samples

Leveraging existing data and samples from past and ongoing trials was identified as a valuable, near-term opportunity. It was strongly encouraged that sponsors retrospectively analyze their extensive datasets for predictive biomarkers. There was a consensus that negative data are as relevant as positive findings, as this prevents redundant research.

A specific proposal was made to analyze serum from patients who experienced adverse events for a specific subcategory of "opsonizing" antibodies that are uniquely capable of facilitating a strong innate immune response when complexed with the AAV vector. This type of antibody was found to be rare among a panel of 30 neutralizing antibodies from SMA patients (Muntoni et al, manuscript in preparation).

It was also noted that analyzing rare severe or fatal outcomes (e.g., three cases of acute liver failure) is statistically challenging due to the high risk of finding irrelevant correlations. A suggested solution is to broaden the analysis to include the much larger group of patients who experienced non-fatal, early-phase liver injury, under the assumption that these are proxy for the more severe outcomes. Underpinning the entire discussion was the acknowledgment that progress requires a coordinated, well-funded, and collaborative effort. It was repeatedly stated that more ambitious projects, such as WGS and systematic, multi-site sample sharing, are impossible without dedicated funding. Grant applications are seen as a mechanism to link forward-thinking ideas with the people and resources needed to execute them.

The formation or continuation of the working groups or a consortium was proposed to develop protocols, write grant applications, and create a framework for collaboration. Significant institutional and governmental hurdles exist for sharing samples and data, especially across international borders. A formal, funded collaboration is seen as the only way to overcome these barriers.

9. Screening, monitoring, and future directions

Chairs: Carsten Bönnemann, Francesco Muntoni and Hildegard Büning

The discussion focused on the pressing need to move beyond current anecdotal practices toward a more systematic, data-driven understanding of adverse events. A primary concern is the emergence of previously under-recognized toxicities, such as capillary leak syndrome, which have been linked to patient deaths. The discussion advocated for leveraging advanced analytical tools, including AI and machine learning, to mine complex datasets for novel biomarkers and predictive signals. While the pathogenesis of some issues, like dorsal root ganglion (DRG) toxicity, is now better understood, systemic inflammatory responses remain a significant challenge.

A comprehensive framework for future research is proposed, emphasizing standardized data collection, the critical review of current protocols, and collaborative data sharing. Finally, the indispensable role of patient organizations is highlighted, not only in funding but in managing patient expectations, communicating risks, and fostering a transparent, collaborative research ecosystem where information is shared back with the community.

9.1. Advanced data analysis

One of the main challenges identified is the inability to predict or fully understand complex immune-mediated toxicities using conventional methods. The discussion strongly advocated for the adoption of sophisticated data analysis techniques to uncover non-intuitive patterns and signals.

In addition of exploring novel biomarkers, the need for expanding the range of biomarkers measured at baseline and post-infusion was emphasized. High-throughput, highly multiplexed proteomics platforms were highlighted as promising tools capable of quantifying thousands of proteins from minimal sample volumes. While access to high-throughput platforms is a barrier for academic labs, the proposed solution is to approach companies with deep experience to request contributions or collaborations, as they have a self-interest in comparing data against their own extensive datasets.

A suggestion was made to systematically measure markers of endothelial damage and dysfunction at baseline and during the first week post-infusion. For example, tests such as von Willebrand factor (VWF) antigen and VWF propeptide to assess TMA were mentioned as being readily available and not overly expensive.

A key proposal involved collecting highly complex, multiplex datasets (e.g., cytokines, clinical outcomes) and applying AI-driven or machine learning tools. The goal is to recognize trends of immune markers that are not preconceived, moving beyond the search for a single biomarker that reaches a certain threshold. While machine learning approaches have been explored, they have so far produced unlikely connections. A significant hurdle is understanding which parameters are required to include in these analytical models. There is a need for expert guidance to identify these parameters.

A substantial opportunity exists in re-analyzing existing data with a specific focus. It was suggested that large datasets, such as those from the Novartis SMART study or from Pfizer, could be analysed retrospectively. For example, the SMART study noted that 70% of patients had a decrease in platelets and over 80% had a rise in transaminases, suggesting that stored blood samples or genomic information from that study could yield valuable insights into inflammatory markers and complement activation.

9.2. Emerging concern: capillary leak syndrome and endothelial health

A significant portion of the discussion focused on capillary leak syndrome as a "new emerging problem" that was not well-anticipated and is linked to severe outcomes, including a patient death published in the New England Journal of Medicine [51] as well as additional reported (yet unpublished) deaths. The consensus is that while it is the role of specialists to engage when problems occur, having considered potential markers and interventions beforehand is critical. When discussing vascular leakage, a question was raised about a potential link to flaviviral infections (e.g., Dengue, West Nile virus), which are known to cause vascular leakage. The mechanism of antibody-mediated enhancement, where pre-existing antibodies worsen a subsequent infection was cited as a parallel. It was suggested to perform serology for these viruses in patients.

A key proposal to monitor endothelial function was to establish baseline measurements of endothelial function and monitor for significant changes, which could then trigger specific interventions. The discussion identified potential therapeutic options used in other conditions

that could be considered for capillary leak syndrome in the context of gene therapy (Table 3).

9.3. Dorsal root ganglion (DRG) toxicity: pathogenesis and mitigation

In contrast to systemic toxicities, the mechanism of DRG toxicity is considered fairly well understood, with NHPs serving as a close predictive model for the human toxicity [59]. The main cause is the high efficiency with which DRG neurons absorb AAV vectors. This leads to a universal form of UPR (unfolded protein response) stress. In most cases, this injury is well-tolerated and asymptomatic, mirroring observations in humans. Studies in NHPs showed that the transgene protein product caused most of the reaction.

Experience has shown that DRG toxicity can be managed by modulating transgene expression. The most successful approach involves pairing a weaker promoter with a microRNA target sequence [60]. This combination reduces expression to a level that avoids toxicity.

For patients receiving intrathecal gene therapy, a clear recommendation was made to standardize monitoring practices. This includes conducting baseline nerve conduction studies and collecting and storing cerebrospinal fluid (CSF) for future analysis of markers like neurofilament light chain profiling, especially if an adverse event occurs.

9.4. A Proposed framework for collaborative research

To address the gaps in knowledge, a structured framework for research and collaboration was outlined. This framework has three main areas:

- 1. Standardized data collection- SOPs: Develop standard operating procedures for data and sample acquisition in three contexts: everyday clinical practice, during an actively developing toxicity, and for specific research questions.
- 2. Critical review of protocols for prevention and intervention: Systematically review current prevention and intervention protocols. This will help define the "lack of knowledge" and propose comparative studies to determine best practices, rather than relying on anecdote.
- 3. Data sharing and analysis infrastructure: Establish a formal framework for data sharing, collection, and analysis, including whole-genome sequencing. This should also feature a working group or forum to rapidly address and disseminate information about emerging toxicities.

Table 3
Potential therapeutic medications/strategies for capillary leak syndrome.

Therapeutic Agent/Class	Notes and Rationale
Defibrotide	An effective drug used in oncology and bone marrow transplant for capillary leak syndrome.
Immune antagonists	Includes anti-complement agents, with the caveat that real-world experience with specific interventions, such as reported here for pegcetacoplan (Danon trial, Rocket Pharma), has been associated with capillary leak syndrome
Antithymocyte Globulin (ATG)	Antibodies that target and deplete T-lymphocytes. Used in organ and bone marrow transplantation to prevent or treat acute rejection.
Canakinumab	An FDA-approved IL-1 inhibitor. IL-1 is the end product of the inflammasome pathway.
Albumin Infusion	A suggestion based on the observation that some cases have had low albumin levels. Infusion could increase intravascular osmotic pressure and draw fluid back into circulation.
IVIg (Intravenous immunoglobulin)	Supported by an anecdote where IVIg led to a quick recovery (within 24 hours) for a patient with viral-induced transaminitis and coagulopathy. The treatment was successful on two separate occasions for the same patient.

A critical warning was raised about the danger of research findings being "overly generalized" by clinicians without deep expertise. Clear guidance on what to look for, what to assess and how to intervene is needed to prevent unrefined or inappropriate treatment decisions.

10. Systematic data collection, data sharing and collaborative platforms

Chair: Genevieve Laforet

Case studies highlighted the critical need for a pre-competitive collaborative approach to data sharing to better understand and mitigate safety risks across the field. However, significant barriers were identified, including sponsor concerns, lack of funding and limited resources within academic registries. Furthermore, a gap exists as many of the key safety biomarkers and data points discussed are not currently collected systematically.

Comparative analysis of shared adverse event cases would allow identification of commonalities, differences, and underlying mechanisms. Collated data can help elucidate patient variability and the role of factors like circulating antagonists or interferon-gamma in treatment responses. A collective dataset can inform the selection of biomarkers, refine inclusion/exclusion criteria, and improve trial design to mitigate risks. It provides a mechanism to archive and analyze data from discontinued or failed trials, ensuring that the information is not lost.

Three main areas of challenge were discussed.

- 1) Concerns by sponsors: legal risks, intellectual property (IP) protection, concerns on a negative impact on public image. Perceived risk to regulatory approval. Hesitancy to contribute disproportionately large datasets compared to other partners.
- 2) Concerns by academics: severe lack of funding and bandwidth for data curation, standardization, and extraction. Academics are often too consumed by grant writing to maintain basic registry operations.
- 3) Data gaps: a key challenge is that many of the critical safety signals being discussed are not currently being collected systematically in registries or natural history studies. A first step circles back to the development of SOPs for prospective data collection.

In response to these challenges, the Critical Path Institute's (C-PATH) Rare Disease Cures Accelerator Data Analytic Platform (RDCA-DAP) was presented by Yanis Mimouni (C-PATH Europe) as a potential solution. This platform offers a secure, centralized, and governed infrastructure for integrating and analyzing patient-level data from diverse sources. Its model is built on controlled sharing, ensuring data owners retain control while enabling powerful, cross-study analysis.

C-PATH is a non-profit organization that builds consortia among industry, academia, regulators, and patient groups to solve drug development challenges in a pre-competitive environment. The RDCA-DAP is a centralized and standardized infrastructure designed to accelerate disease characterization and drug development by promoting patient-level data sharing. Their aim is to achieve official regulatory endorsement from bodies like the FDA and EMA for novel methodologies and drug development tools developed through the platform. The presentation of the C-PATH platform prompted a pragmatic discussion about implementation, sponsor engagement, and the path forward.

Sponsors confirmed their engagement: Pfizer confirmed its conceptual commitment to data sharing and is actively working with C-PATH on a data request from its discontinued Duchenne program. The process is noted to be complex due to internal governance and international data privacy laws. Regenxbio is using the platform to support an external natural history control group.

The challenge of funding for academic registries to prepare and contribute data remains a critical hurdle. The potential for European funding mechanisms was discussed as a possible solution.

The discussion concluded in exploring collaborations with C-PATH and with a consensus that the immediate, critical next step is for the

established working groups to develop standardized operating procedures (SOPs) for the collection of essential safety data to create a harmonized, high-quality dataset for future collaborative analysis.

The proposed "pre-competitive task force" was seen as a straightforward, project-based approach to initiate collaboration.

The meeting concluded with a clear and actionable path forward.

1. Develop Data Collection SOPs: The immediate priority and next deliverable for the working groups is to define and develop SOPs for collecting the relevant safety data that is currently missing. This should include basic, feasible, and advanced tiers of data collection.
2. Harmonize Data: The ultimate goal of developing SOPs is to ensure that future data is collected in a harmonized and interchangeable manner across different studies and registries.
3. Continue Collaboration: There was strong agreement on the success of the meeting and the need to keep the working groups active and maintain the collaborative momentum, with plans to convene follow-up meetings at future scientific conferences.

Organizers

Carsten G. Bönnemann, Hildegard Büning, Francesco Muntoni

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Funding

This workshop was supported by Parent Project Muscular Dystrophy (PPMD).

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Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Given their role as editorial board members, RO and FM had no involvement in the review of this article and had no access to information regarding its review. Full responsibility for the editorial process for this article was delegated to another journal editor. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The workshops and next generation programme are made possible

thanks to the financial support of the European Neuromuscular Centre (ENMC) and its Full Partners: Association Française contre les Myopathies (France), Deutsche Gesellschaft für Muskelkranke (Germany), Muscular Dystrophy Campaign (UK), Muskelsvindfonden (Denmark), Prinses Beatrix Spierfonds (The Netherlands), Schweizerische Stiftung für die Erforschung der Muskelkrankheiten (Switzerland), Spierziekten Nederland (The Netherlands), Telethon Foundation (Italy). In addition, we would like to thank the Associated Partners: FSHD Europe, Österreichische Muskelforschung (Austria), SMA Europe, TREAT-NMD, World Duchenne Organisation, World Muscle Society (WMS), and the members of the ENMC Company Forum: Amicus Therapeutics, Avidity Biosciences, Argenx, Dyne Therapeutics, Lupin Neuroscience, Novartis, Roche, Sanofi, Santhera and Sarepta.

Acknowledgment of all participants who contributed to pre-meeting virtual working groups discussions (Alphabetical order; chair names are bolded):

Anti-transgene: Basel Assaf; Carsten Bönnemann; **Serge Braun;** Hildegard Büning; Barry Byrne; **Jeffrey Chamberlain;** Jahannaz Dastgir; Kevin Flanigan; Monica Rebeca Gil Garzon; Bradley Hamilton; Sharon Hesterlee; Janbernd Kirschner; Dan Levy; Francesco Muntoni; Alexander Murphy; Rotem Orbach; Kanneboyina Nagaraju; Isabelle Richard; Giuseppe Ronzitti; Ulrike Schara-Schmidt.

TMA: Basel Assaf; Carsten Bönnemann; Gabriel Brooks; Hildegard Büning; Barry Byrne; Jahannaz Dastgir; **Kevin Flanigan;** Monica Rebeca Gil Garzon; Bradley Hamilton; Juliette Hordeaux; Anna Kajaste; **Genevieve (Jenny) Laforet;** Dan Levy; Ronit Mazar; Hugh McMillan; Francesco Muntoni; Rotem Orbach; Giuseppe Ronzitti; Jonathan Schwartz; Qi Shen

Liver: Basel Assaf; Carsten Bönnemann; Bradley Hamilton; Hildegard Büning; Jahannaz Dastgir; **Anil Dhawan;** Monica Rebeca Gil Garzon; Juliette Hordeaux; **Rohit Kohli;** Hugh McMillan; Francesco Muntoni; Alexander Murphy; Rotem Orbach; James Richardson; Giuseppe Ronzitti; Violeta Stoyanova-Beninska

Cardiac: Basel Assaf; Carsten Bönnemann; Gabriel Brooks; **Barry Byrne;** Jahannaz Dastgir; Pat Furlong; Monica Rebeca Gil Garzon; Bradley Hamilton; Anna Kajaste; Dan Levy; Francesco Muntoni; Rotem Orbach; Rosaline Quinlivan; Sandra Reyna; Isabelle Richard; James Richardson; Ulrike Schara-Schmidt; Jonathan Schwartz; Qi Shen; **Karim Wahbi**

Graphical abstract was created in BioRender. Orbach, R. (2026) <https://BioRender.com/ij5zirl>

During the preparation of this work, the authors used NotebookLM (google) and Claude (Anthropic, Claude Sonnet 4.5) to assist with drafting of the meeting report. The authors reviewed and edited the output as needed and take full responsibility for the content of the published article.

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