Accepted Manuscript

Submission Date: 2024-02-02
Accepted Date: 2024-08-04
Accepted Manuscript online: 2024-09-11

Thrombosis and Haemostasis

Emerging thrombotic disorders associated with virus-based innovative therapies: from VITT to AAV-gene therapy-related thrombotic microangiopathy

Silvia Benemei, Francesca Gatto, Rossella Marcucci, Paolo Gresele.

Affiliations below.

DOI: 10.1055/a-2413-4345

Please cite this article as: Benemei S, Gatto F, Marcucci R et al. Emerging thrombotic disorders associated with virus-based innovative therapies: from VITT to AAV-gene therapy-related thrombotic microangiopathy. Thromb Haemost 2024. doi: 10.1055/a-2413-4345

Conflict of Interest: Professor RM and Professor PG were paid consultants to Pfizer in connection with the development of this manuscript.
SB and FG are Pfizer employees.

Abstract:
Gene therapy is a therapeutic approach for treating life-threatening disorders. Despite the clinical improvements observed with gene therapy, immune responses either innate or adaptive against the vector used for gene delivery can affect treatment efficacy and lead to adverse reactions. Thrombotic microangiopathy (TMA) is a thrombosis with thrombocytopenia syndrome (TTS) characterized by microangiopathic hemolytic anemia, thrombocytopenia, and small vessel occlusion known to be elicited by several drugs that has been reported as an adverse event of adeno-associated virus (AAV)-gene therapy. TMA encompasses a heterogenous group of disorders, its classification and underlining mechanisms are still uncertain, and lacks validated biomarkers. The identification of predictors of TMA, such as vector dose and patient characteristics, is a pressing need to recognize patients at risk before and after AAV-based gene therapy administration.
This review aims to explore the literature on TMA associated with AAV-based gene therapy in the context of TMA (i.e., hemolytic-uremic syndrome, thrombotic thrombocytopenic purpura and other drug-related TMAs). Considering the wide attention recently gained by another TTS associated with a non-gene therapy viral platform (adenovirus, AV COVID-19 vaccine), namely vaccine-induced immune thrombocytopenia and thrombosis (VITT), AAV gene therapy-related TMA mechanisms will be discussed and differentiated from those of VITT to avoid recency bias and favor a correct positioning of these two recently emerged syndromes within the heterogenous group of drug-related TTS. The review will discuss strategies for enhancing the safety and optimize the management of AAV-based gene therapy, emerging as an efficacious therapeutic option for disparate, severe, and often orphan condition.

Corresponding Author:
Dr. Silvia Benemei, Pfizer Italy, Via Valbondione, 00188 Roma, Italy, silvia.benemei@pfizer.com

Affiliations:
Silvia Benemei, Pfizer Italy, Roma, Italy
Francesca Gatto, Pfizer Italia Srl, Roma, Italy
Rossella Marcucci, University of Florence, Medical and Surgical Critical Care Medicine, Florence, Italy
Paolo Gresele, University of Perugia, Department of Medicine, Division of Internal and Cardiovascular Medicine, Perugia, Italy

Emerging thrombotic disorders associated with virus-based innovative therapies: from VITT to AAV-gene therapy-related thrombotic microangiopathy

Silvia Benemei[1], Francesca Gatto[1], Rossella Marcucci[2*], Paolo Gresele[3*]

[1] Medical Affairs, Pfizer Italia, Italy

[2] Department of Experimental and Clinical Medicine, University of Florence and Azienda Ospedaliero-Universitaria Careggi, Firenze, Italy

[3] Section of Internal and Cardiovascular Medicine, Department of Medicine, University of Perugia, Perugia, Italy

*share senior authorship

Corresponding author:

Silvia Benemei

Email: silvia.benemei@pfizer.com

Abstract

Gene therapy is a promising therapeutic approach for treating life-threatening disorders. Despite the clinical improvements observed with gene therapy, immune responses either innate or adaptive against the vector used for gene delivery can affect treatment efficacy and lead to adverse reactions. Thrombotic microangiopathy (TMA) is a thrombosis with thrombocytopenia syndrome (TTS) characterized by microangiopathic hemolytic anemia, thrombocytopenia, and small vessel occlusion

known to be elicited by several drugs that has been recently reported as an adverse event of adeno-associated virus (AAV)-gene therapy. TMA encompasses a heterogenous group of disorders, its classification and underlining mechanisms are still uncertain, and still lacks validated biomarkers. The identification of predictors of TMA, such as vector dose and patient characteristics, is a pressing need to recognize patients at risk before and after AAV-based gene therapy administration. This review aims to explore the literature on TMA associated with AAV-based gene therapy in the larger context of TMA (i.e., hemolytic-uremic syndrome, thrombotic thrombocytopenic purpura and other drug-related TMAs). Considering the wide attention recently gained by another TTS associated with a non-gene therapy viral platform (adenovirus, AV COVID-19 vaccine), namely vaccine-induced immune thrombocytopenia and thrombosis (VITT), AAV gene therapy-related TMA mechanisms will be discussed and differentiated from those of VITT in order to avoid recency bias and favor a correct positioning of these two recently emerged syndromes within the heterogenous group of drug-related TTS. Finally, the review will discuss strategies for enhancing the safety and optimize the management of AAV-based gene therapy that is emerging as an efficacious therapeutic option for disparate, severe, and often orphan conditions.

Keywords

Adeno-associated virus (AAV), biomarkers, gene therapy, thrombotic microangiopathy (TMA), vaccine-induced immune thrombocytopenia and thrombosis (VITT)

Introduction

Gene therapy involves the administration of genetic material into a patient's body to modify specific cell functions. This methodology encompasses various approaches, such as gene replacement, silencing, editing, and addition.[1]

Replacement gene therapy delivers a functional gene to substitute for the endogenous gene carrying a loss-of-function mutation. Considerable progress has been achieved in recent years in replacement gene therapy with encouraging results for the treatment of monogenic life-threatening or highly incapacitating disorders. So far, replacement gene therapies that have been granted marketing authorization by the European Medicine Agency (EMA) and the Food and Drug Administration (FDA), include alipogene tiparvovec for patients with lipoprotein lipase deficiency (withdrawn by EMA in 2017 due to lack of demand), voretigene neparvovec-rzyl for Leber's congenital amaurosis, onasemnogene abeparvovec for spinal muscular atrophy (SMA), fidanacogene elaparvovec (approved by FDA) and etranacogene dezaparvovec (conditionally approved by the EMA) for hemophilia B, and valoctocogene roxaparvovec for hemophilia A (conditionally approved by the EMA).[2] All these gene therapies rely on recombinant adeno-associated virus (AAV) as vector for gene delivery highlighting the versatility and usefulness of this viral vector platform.[1, 3, 4] Moreover, several clinical trials evaluating AAV-based gene therapies are currently ongoing. A search of the database clinicaltrials.gov performed on April 20, 2024, with the keyword "AAV" identified 116 active (recruiting or non-recruiting) studies, including trials on diseases such as Parkinson disease, age-related macular degeneration, and Fabry's disease.

AAVs are non-enveloped viruses that rely on other viruses for their replication and, although they can infect humans, have not been linked to any human diseases.[1] Several favorable characteristics of AAVs have led to the development of AAV-based vectors for gene therapy, like mild immunogenicity in humans, wide-ranging tropism, virtually no integration in the host genome, prolonged permanence in the host as nuclear extrachromosomal episomes and long-lasting gene expression.[1, 5, 6] Despite these advantages over other viral vectors, and the successful development of

several gene therapies, AAV-based gene delivery is not without limitations. In fact, beside the considerable improvements obtained with gene therapies with an overall positive impact on patients' clinical outcomes and quality of life,[7] safety issues must be carefully appraised.[8, 9] Immune responses against the vector remain a major challenge as they can reduce treatment efficacy and cause adverse reactions.[5, 10, 11] In particular, adaptive immune responses against the virus capsid and the transgene product can impair replacement gene-therapy by reducing the effectiveness of cell transduction with the AAV-vector and the amount of available transgene product. At the same time, innate immunity involving the complement system is increasingly implicated in some severe adverse events, including thrombotic microangiopathy (TMA) reported in patients treated with gene therapy.[5, 10, 11] In clinical trials of AAV gene therapy severe treatment-related adverse events have been reported with a rate of 30.6%, according to a meta-analysis of 255 studies.[12] These events included hepatotoxicity, dorsal root ganglia toxicity, myocarditis, and TMA.[6, 10, 12] In particular, hepatotoxicity and TMA are consistently associated with systemic AVV gene therapies[6, 12] and appear to be dependent on AAV-vector doses and to the route of administration (i.e., tissue-specific responses at the site of injection, systemic immune responses in case of intravenous injection).[10] Predictors of severe adverse events have yet to be established, however possible candidates include gene therapy characteristics, such as vector dose, and patient characteristics, such as preexisting immunity to AAV, disease stage, body weight.[6]

TMA is an overarching term used to describe a disease process as well as a heterogeneous group of rare disorders characterized by vessel occlusion, thrombocytopenia, and microangiopathic hemolytic anemia,[13-16] the latter being the hallmark of this condition. In particular, microangiopathic hemolytic anemia is characterized by many schistocytes and red blood cells fragments, high lactate dehydrogenase levels, and low/absent haptoglobin. TMA, regardless of its etiology, is a hematologic emergency that requires urgent treatment being associated with considerable morbidity and mortality.[17-19] The classification of TMA is complex and continuously evolving and, so far, no

shared and consistent terminology is available for defining the various members of the TMA family.[20] Drug-related TMA may be included in the larger group of thrombosis with thrombocytopenia syndrome (TTS), which encompasses a heterogenous group of immune and non-immune conditions including cancer, thrombotic thrombocytopenic purpura, disseminated intravascular coagulation and antiphospholipid antibody syndrome.[21] TTS diagnosis is not based on specific test results and is readily applicable also to low-resource settings,[22] thus it is especially used by the WHO and regulatory agencies in the context of pharmacovigilance. Recently, a new disease called vaccine-induced immune thrombocytopenia and thrombosis (VITT) has been identified and included among the immune-mediated TTS. [23, 24] This novel syndrome emerged in March 2021 at the beginning of the vaccination campaign against coronavirus disease 2019 (COVID-19), and was reassociated with the administration of the first dose of the adenovirus (AV)-based vaccines ChAdOx1 nCoV-19 and Ad26.COV2.S against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2).[25-27]

This article aims to review current literature about TMA-related adverse events associated with AAV-based gene therapy, with the ultimate goal of discussing strategies to optimize the safety of this innovative and promising therapeutic approach. The review will first focus on TMA characteristics and on reports of TMA associated with AAV-based gene therapy. Then it will discuss the proposed mechanisms responsible for these events, comparing them with those leading to VITT, given some similarities between these two syndromes, to properly position them in the heterogenous group of drug-related TTS. However, despite some “similarities”, VITT is clearly not a TMA disorder. Heparin-induced thrombocytopenia (HIT) and VITT typically cause large-vessel thromboses involving veins and arteries, whereas organ dysfunction in TMA typically involves microvascular platelet-rich microthrombi. The existence and influence of cognitive bias in medicine is a well-known topic and is gaining increased attention.[28, 29] Given that “recency bias” may lead to choosing a particular interpretation or diagnosis because it is at the front of mind [29] it is worth

considering that AAV gene therapy-related thrombotic disorders may be confused for VITT by non-experts in coagulation disorders, considering that the latter has dominated the drug safety literature in the last few years. Accordingly, a clarification of the specific characteristics and mechanisms of the two distinct adverse events related to these innovative but different virus-based platforms, namely AAV for gene-therapy and AV for the COVID19 vaccine, may be useful to provide the knowledge and rationale for appropriate clinical management. Table 1 provides a summary of the main TTS discussed in the present review, along with their clinical characteristics and diagnostic criteria, classifying them as being a TMA or not. Finally, potential biomarkers for TMA diagnosis and for the identification of patients at risk of TMA will also be addressed.

Classification of thrombotic microangiopathies

An established classification of TMA disorders is lacking, according to the complexity of the scenario and its rapid evolution. Clinically, TMA disorders are characterized by thrombocytopenia, microangiopathic hemolytic anemia, and brain and kidney injury, although injury to any other organ may also occur[14, 15] (Visual summary). Thrombocytopenia arises from the consumption of platelets into microcirculatory platelet-rich thrombi, while microangiopathic hemolytic anemia is caused by the fragmentation of red blood cells in the occluded microvasculature.[15] Kidney injury is frequent, as this organ is particularly vulnerable to microvascular occlusion and endothelial dysfunction.[13] Two members of the TMA group with these characteristics – thrombotic thrombocytopenic purpura (TTP) and the hemolytic-uremic syndrome (HUS) – have been known for decades.[14] The pathogenic mechanisms underlying TTP and HUS were discovered in the early ‘80s, namely the inability to degrade ultra-large multimers of von Willebrand factor (VWF) for TTP, and the exposure to the Shiga toxin produced by *Escherichia coli* leading endothelial damage for HUS.[30, 31] Acquired and congenital TTP are due primarily to severe ADAMTS 13 deficiency, atypical HUS (aHUS) is commonly associated with complement dysregulation, and Shiga toxin, drugs, immune complexes, and others agents likely damaging the endothelium. Impaired post-secretion processing

of VWF due to deficiency of ADAMTS 13 (IgG antibodies or congenital), dysregulation of the alternative complement pathway (mutations and/or specific antibodies), and endothelial injury are pathophysiologic mechanisms involved in these TMAs.[14]

TMA disorders are currently divided into primary and secondary forms. Primary TMA are associated to genetic or acquired factors related to specific mechanisms (i.e., low or undetectable ADAMST 13 activity for TTP, permanent complement activation for aHUS); while in secondary forms, other diseases or drugs, by different mechanisms, cause the activation of complement [10, 32] finally leading to microvascular endothelial injury (e.g., AAV-associated TMA).

Drug-induced TMA has been reported in association with several medicinal products.[33] A systematic review of 344 reports involving a total of 586 patients identified 78 drugs implicated in drug-induced TMA.[34] Of these, 22 (28%) were conclusively demonstrated as causing TMA, while 20 (26%) were likely associated with TMA.[35] Clinical presentation of drug-induced TMA is variable, including organ damage, neurological signs and ischemia, suggesting different pathogenic mechanisms which can involve a direct toxic effect or an immune-mediated effect.[13] In immune-mediated drug-induced TMA, disease onset is usually acute and drug-dependent antibody testing may help identify the causative agent. In non-immune drug-induced TMA, the onset is usually gradual and dose-dependent.[20]

In a review article on the mechanisms of TMA published some years ago, three types of microangiopathy were recognized based on histopathologic features: TMA with systemic platelet thrombi (for example TTP), TMA with predominantly renal platelet-fibrin thrombi (for example classic and familial HUS), and TMA with renal or systemic thrombi (for example, drug-induced TMA).[14]

TMA associated with AAV-based gene therapy

We searched PubMed for “TMA AND [adeno-associated virus OR AAV]” and “thrombosis AND [adeno-associated virus OR AAV]” and “[TMA OR thrombosis] AND gene therapy”, selecting case reports or case report reviews describing the adverse event of interest up to March 2024.

There is still very limited information concerning TMA in patients receiving AAV-based gene therapy. This is primarily due to the small number of patients enrolled in clinical trials evaluating gene therapy for the treatment of rare diseases together with the very low incidence of this adverse event. With respect to the reporting of adverse events related to gene therapy [e.g., for the treatment of Duchenne muscular dystrophy (DMD)], substantial efforts towards prompt disclosure have been made by pharmaceutical companies producing AAV-based gene therapies in compliance with the request of regulatory authorities and safety issues have been addressed, whenever possible, in a multidisciplinary approach, with the engagement of the scientific community.[36]

TMA has been initially reported with gene therapy products that use the vector serotype AAV9 delivered systemically and at high doses ($\geq 5 \times 10^{13}$ vector genomes/kg body weight), for the treatment of neuromuscular disorders in children:[6] onasemnogene abeparvovec approved for the treatment of SMA, and SGT-001 and PF-06939926 under investigation for the treatment of DMD[6, 33] [NCT03368742, NCT03362502]. For two patients (one treated with onasemnogene abeparvovec and the other with SGT-001), TMA was fatal.[10, 20, 37, 38] So far, no TMA-related events have been associated with voretigene neparvovec-rzyl for the treatment of inherited retinal dystrophy, which uses a AAV2 vector and is delivered via a subretinal injection.[6]

TMA in children is a rare event (approximately 1.0 – 3.3 cases/million/year).[33] According to an FDA report published in 2021 and addressing AAV-related TMA, nine cases of TMA were reported over more than 1400 patients treated with onasemnogene abeparvovec; these cases involved children aged between 4 months and 4 years.[33] In four published case reports, TMA symptoms started shortly after the beginning of treatment (at day 8) and the patients had hematologic markers of complement activation (low C3 and C4 and/or increased levels of soluble C5b-9 complex).[33, 37, 39]

More recently, complement activation has been reported in adult subjects with Fabry's disease receiving one single dose of 4D-310 (AAV2 capsid variant) in combination with prophylactic oral corticosteroids, thus suggesting that these phenomena are likely not AAV9 specific.[40] In the majority of cases, TMA eventually resolved after management including fluid and electrolyte infusion, platelet or red blood cell transfusion, dialysis, plasmapheresis, and eculizumab,[33] while one case was fatal (supplementary table S1).[37]

Importantly, in order to optimize the gene therapy safety profile, several preventive/therapeutic strategies targeting the immunologic response to AAV have been proposed, including a) high-dose glucocorticoids; b) anti-CD20 mAbs that, by the depletion of B cell populations, impair antibody production over time; c) mTOR inhibitors that contribute to the inhibition of T and B cell activation; 4) plasmapheresis and 5) cleavage of circulating IgG antibodies.[41, 42]

Possible mechanisms of TMA associated with AAV-based gene therapy

The mechanisms leading to the TMA related-events reported with AAV-based gene therapy are not fully elucidated. Recent research work has focused on the role of the innate immune system in the response to AAV-based gene therapy.[43] The involvement of the innate immune system has been suggested by the consistent evidence of complement-mediated events in the few reports of TMA discussed above.[20, 33, 37] Complement may be activated by the classical (Ig-mediated) and alternative pathways, which converge to produce the C3 convertases that cleave C3 into C3a and C3b, the latter binding C4b2b to create C5 convertase. The C5 convertase produces C5a, a very potent inflammation mediator, and C5b, which in turn binds to C6, C7, C8, and C9 to generate the C5b-9 membrane attack complex mediating cell death.[44] Complement pathways, which are components of the innate immune system, protect against viruses and can interact with the adaptive immune system. Activation of these pathways initiates a cascade of events – inflammatory response, recruitment of neutrophils, stimulation of coagulation, endothelial cell damage, and platelet activation – that eventually result in injury to the microvasculature and thrombosis.[10] Innate immune

responses are rapid (within a few days to 2 weeks from exposure to the virus, as observed in the few reports of TMA in patients treated with AAV-gene therapy), while antigen-specific adaptive immune responses develop usually after several weeks.

A few studies have attempted to dissect the pathogenesis of TMA related to AAV-gene therapy.[43, 45] Detailed studies on TMA mechanisms are still lacking, also because in animal models a direct involvement of the complement system in AAV-mediated events has not clearly emerged.[4] Importantly, it has been demonstrated that AAV capsid particles interact with some complement proteins (namely C3, C3b, iC3b) and complement regulatory factor H.[41] More recently, to bypass possible species differences, Smith et al. have performed studies using human whole blood and have characterized the innate immune response to AAV.[43] They found that AAV particles were mainly internalized by neutrophils, monocyte-related dendritic cells, and monocytes. Low titers (≤ 1:10) of pre-existing AAV neutralizing antibodies had a negligible effect on the innate immune response to AAV, while higher titers (≥ 1:100) were associated with a significant increase in the secretion of proinflammatory cytokines, AAV-vector uptake by antigen presenting cells, and complement activation. The results of this study suggest that preexisting antibodies to AAV vectors may play a crucial role in the immunogenicity of AAV-based gene therapies and that inhibition of the complement pathway may be a strategy for improving the safety of these therapeutics.[43] West et al. investigated complement activation by AAV9 in sera from seronegative and seropositive (i.e., carrying neutralizing antibodies from a previous environmental exposure to AAV) human donors.[45] The interaction of AAV9 with seropositive sera resulted in complement activation; again, complement activation was associated with the sera that had higher levels of anti-AAV IgG1 antibodies. These findings suggest that immunoglobulin depletion might be a strategy for reducing the risk of complement activation associated with AAV-based gene therapy.[45] More importantly, clinical data have been recently published in support of the involvement of complement activation, corroborating its possible targeting for therapeutic purposes. Byrne et al. reported that, after the

systemic AAV9 administration to deliver gene therapies, subjects treated with only corticosteroids (n=13) showed an increase of IgM, IgG, D-dimer, a decline in platelet count and direct and alternative complement activation (depletion of C4, elevated SC5b-9, Ba, and Bb antigens). On the other hand, subjects treated with corticosteroids plus rituximab and sirolimus (n=25), did not show significant changes in either the Ig profile or in complement levels.[40] Hence, despite some limitations, such as the absence of randomization and the heterogeneity of clinical diagnoses, the study has shown that TMA in the setting of AAV gene therapy is primarily due to complement activation by the classical (antibody-dependent) pathway, possibly amplified by the alternative pathway.

Does AAV gene-therapy-related TMA share mechanisms with VITT and other immune-mediated TTS?

The emergence of TMA associated with AV-based anti-SARS-CoV-2 vaccines has prompted intensive research on the pathogenic mechanisms of thee thrombotic events.[23, 46-49] Currently, it does not seem possible to completely exclude that VITT and AAV-vector-related TMA may share some features, and the efforts towards the understanding of VITT may contribute to improve current knowledge of the mechanisms leading to severe events in patients treated with AAV-based gene therapy, at least as a reference model from which the latter differentiate.

The evolution of VITT denomination is a clear example of how complex is the classification of thrombotic disorders, even in the current era where disease nomenclature has progressively increased its role in medicine.[49] Indeed, while the first paper reporting the syndrome used "thrombotic thrombocytopenia" in the title, this name was later considered misleading as VITT is not a TMA, i.e., schistocytes are absent and, if present, in keeping with an underlying disseminated intravascular coagulation (DIC). Accordingly, most of the subsequent papers adopted the same acronym but as "vaccine-induced immune thrombocytopenia and thrombosis", paralleling "heparin-

induced thrombocytopenia and thrombosis" (HIT/T),[50] which also is not a TMA. In addition, HIT and VITT typically cause large-vessel thromboses involving veins and arteries, whereas organ dysfunction in TMA disorders is typically the consequence of microvascular-platelet-rich microthrombi.

Although considerable progress has been made in the understanding of VITT, the suggested pathogenesis remains, at least in part, unclarified.[20, 27, 49, 51, 52] VITT has been classified in the larger group of "platelet activating anti- platelet factor 4 (PF4) disorders" including diseases characterized by a) thrombocytopenia and thrombosis; b) circulating anti-PF4 antibodies; c) a short time interval from the initial exposure to the trigger (heparin; adenovirus) to clinical manifestations; d) clotting activation mediated not only by the activation of platelets, but also of monocytes, neutrophils and endothelium.[53] Patients with VITT present antibodies directed to PF4 which are pathogenic and induce platelet activation and the formation of neutrophil extracellular traps leading to thrombosis and thrombocytopenia.[48] The formation of anti-PF4 antibodies is thought to be caused by the presence of PF4-vaccine complexes which, along with the proinflammatory environment caused by vaccination, can trigger a B cell response.[48] According to this view, VITT is a condition similar to autoimmune HIT, a potentially devastating reaction that can occur also without previous exposure to heparin and is caused by antibodies against PF4,[27, 48] although the target antigen(s) on PF4 are different between HIT and VITT antibodies.[53]

It has been recently suggested to use the term "autoimmune HIT" (aHIT) in situations where heparin is the initiating trigger, but in which the resulting antibodies can strongly activate platelets even in the absence of heparin while HIT-like disorders triggered in the absence of heparin should be called "spontaneous HIT" (SpHIT).[54] Importantly both HIT-like and VITT-like antibodies can be triggered in the absence of proximate heparin or vaccine exposure.[55, 56] It is worth mentioning that adenovirus infections had already been reported to be associated with thrombotic disorders,[57] but that recently it has become apparent that they can trigger a VITT-like syndrome characterized by

anti-PF4 antibodies with platelet-activating properties, severe thrombocytopenia, extreme D-dimer elevation, hypofibrinogenemia and multiple atypical thromboses, including CVST.[56, 58-60] Thus, PF4/polyanion ELISAs are considered a good screening test for both HIT/HIT-like and VITT/VITT-like antibodies.[61]

PF4 is a soluble protein that can form dimers and tetramers. Positively charged PF4-mers avidly bind to negatively charged heparin (a polymeric anion). PF4-heparin complexes undergo conformational changes and can become ultra-large and highly immunogenic.[48] In HIT, antibodies to PF4-heparin complexes can activate platelets via the binding to FcγRIIA receptors, leading to the formation of microthrombi and large-vessel thrombosis.[49, 51, 62] It is hypothesized that the VITT counterpart of the highly immunogenic PF4-heparin complexes seen in HIT are complexes of PF4 with DNA and polyadenylated hexon proteins of the AV vector that could come in contact with blood during vaccine administration.[20, 49, 62] Both vector components could provide a scaffold of negative charges similar to heparin.

In conclusion, AV-vectors can induce an immunologic reaction that results in the production of high-titer anti-PF4 antibodies; these, in turn, cause the thrombotic events associated with VITT. An interesting question that deserves further investigation is which additional anionic molecules may be involved in the formation of complexes with PF4, besides those already identified.[52, 63, 64] However, until today no evidence has been collected about the possible involvement of these mechanisms (i.e. anti-PF4 antibodies with strong platelet-activating properties, properly assessed by experienced reference labs) in the TMA related to AAV gene therapy, in agreement with the different mechanism of action, with the obvious consequences for possible mechanism-driven therapeutic options.

Biomarkers

Currently no biomarkers to identify patients at risk have been validated for either TMA or VITT. It should however be pointed out that while biomarkers related to an increased risk for a severe

adverse event would be crucial for small populations of patients candidate to therapy, like those potentially treatable by gene-therapy approaches, they would not be cost-effective to screen thousands of healthy subjects candidate to vaccination. On the other hand, biomarkers useful for TTS diagnosis and monitoring would have similar relevance in the two clinical settings. A number of useful laboratory parameters have been identified. For example, plasma ADAMTS 13 activity measurements have been shown to be essential not only for the initial diagnosis of immune TTP, but also for risk stratification and prompt detection of disease progression or relapse.[65] Of note, thrombocytopenia and platelet activation are characteristic of many different TTS conditions, highlighting the need for disease-specific tools for the assessment and monitoring of changes in platelet activation and function in different settings. Increased soluble C5b9 has been reported in complement-related diseases, including C3 glomerulonephritis and aHUS, and has been proposed as a biomarker of disease progression in HUS and TMA associated with hematopoietic stem cell transplantation.[37]

In the field of gene therapy, there is an urgent and widely recognized need for biomarkers to identify patients at high risk of TMA complications before initiating AAV-based gene therapy, and to ensure the prompt recognition and diagnosis of TMA following therapy delivery.[36, 40, 53, 62, 66] Predisposing factors for TMA to be considered before initiating gene therapy include infections, anti-factor H antibodies, defects in ADAMTS 13 activity, genetic susceptibility to HUS, increased levels of sC5b9 (a biomarker of complement activation).[36] Patients with biallelic null mutations in the gene to be replaced and no cross-reactive immunologic material (i.e., CRIM-negative) are naïve to the transgene product and have a high risk of developing an immune response to it due to the lack of immune tolerance; this could lead to adverse events and ineffective therapy.[37] Notably, none of these factors have so far been validated for the selection of candidates to gene therapy. Importantly, the total anti-AAV Ab levels should be measured before and after AAV gene therapy administration,

because assessing only the neutralizing antibodies provides scarce information about the total amount of complement activating antibodies (e.g. IgM).[67]

In the post-treatment setting, current safety indications about gene therapy issued by the EMA recommend stringent monitoring of platelet count in the weeks following therapy delivery.[66] Screening for hemolysis after any decrease in platelet counts is also recommended. Troponin increases, uremia, increased D-dimers (suggesting blood clotting activation), anemia and schistocytes on the blood smear should also be assessed.[6] In case of clinical suspicion, anti-PF4 antibody assays (PF4/polyanion ELISAs) should be performed to rule out HIT/VITT.[61] The recent clinical data from Byrne and colleagues, reaffirm the relevance of frequent immunosurveillance during the first 30 days post-AAV administration, including not only D-dimer, but also comprehensive complement and hematology panels, in addition to other biomarkers of endothelial activation.[40]

Discussion

Replacement gene therapy is a valuable approach to the treatment of rare, life-threatening conditions for which no other therapeutic options are available. In recent years the progress with AAV-based gene therapy has been considerable leading to the approval of several gene therapies. As with any other treatment, replacement gene therapy is associated with possible adverse events, and the assessment of individual risk-benefit balance should be considered. The reported adverse events, and in particular TMA, appear to be due to the complex set of immune responses elicited by the AAV-vector.

Evidence concerning AAV-related TMA is currently very limited, although increasingly emerging. There is therefore an urgent need to improve and further encourage case reporting and data sharing among clinicians.

Elucidating the mechanisms leading to AAV-related adverse events is crucial for implementing preventive measures, identifying patients at risk (biomarkers needed), adjusting therapy (dosage,

improving vectors towards a more effective and safer gene delivery), and managing the adverse event when not preventable. The identification of the complement system as a crucial player in TMA has important therapeutic implications for patients receiving AAV-based gene therapy due to the availability of already validated complement inhibitors and other drugs able to indirectly prevent its activation.[40]

Although TMA is a rare condition, it can occur in many settings and its consequences can be devastating. There is a need to increase the awareness of these disorders. To this purpose, nomenclature and classification should be consolidated and standardized. Consistent safety monitoring guidelines are required as well.

Conclusions

AAV-gene therapy is fundamental for people, very often children, affected by rare, fatal or highly incapacitating conditions. The potential benefits of this therapeutic strategy are relevant. Additional efforts directed to the further improvement of AAV-based replacement gene therapy are needed.

To enhance the safety and effectiveness of AAV-gene therapies, it is crucial to implement a comprehensive approach addressing the complexities associated with treatment-induced TMA. First and foremost, a concerted effort to improve awareness and education about TMA among healthcare providers and researchers is needed. A pivotal step in this direction involves establishing a clear and universally accepted nomenclature and classification system for TMA, including disorders associated with AAV-gene therapy. Concurrently, the development of shared guidelines for the diagnosis and management of AAV-induced TMA is imperative. These guidelines should encompass safety monitoring protocols and emphasize the implementation of immune-monitoring for patients undergoing AAV-gene therapy.[11] Additionally, further research should be directed towards understanding the intricate mechanisms behind AAV-induced TMA. This involves exploring the underlying biological processes that lead to TMA and searching for relevant biomarkers that can aid in diagnosis and risk assessment. Moreover, it is essential to focus on the

development of innovative strategies aimed at addressing the diverse issues related to AAV immunity.[5, 68] Importantly, recent evidence reinforce initial data about the existence of already available preventive/treatment options that may be safely used to prevent TMA.[40, 43] By collectively pursuing these initiatives, the scientific community can optimize the administration and follow up of AAV-gene therapies, ultimately benefiting patients worldwide.

Authors' Contribution

All authors conceived the project, wrote, edited and revised the manuscript critically. All authors approved the final version of the manuscript.

Conflict of Interest

Professor RM and Professor PG were paid consultants to Pfizer in connection with the development of this manuscript.

SB and FG are Pfizer employees.

Acknowledgments

Medical writing support was provided by Lorenza Lanini at Health Publishing & Services Srl and was funded by Pfizer. Editorial support was provided by Barbara Bartolini at Health Publishing & Services Srl and was funded by Pfizer.

References

1. Bulcha JT, Wang Y, Ma H, Tai PWL, Gao G. Viral vector platforms within the gene therapy landscape. *Signal Transduct Target Ther*. Feb 8 2021;6(1):53. doi:10.1038/s41392-021-00487-6

2. FDA. Approved Cellular and Gene Therapy Products. Accessed 10 May, 2024. https://www.fda.gov/vaccines-blood-biologics/cellular-gene-therapy-products/approved-cellular-and-gene-therapy-products

3. Wang D, Tai PWL, Gao G. Adeno-associated virus vector as a platform for gene therapy delivery. *Nat Rev Drug Discov*. May 2019;18(5):358-378. doi:10.1038/s41573-019-0012-9

4. Bradbury A, Markusic D, Muhuri M, Ou L. Editorial: Immunogenicity and toxicity of AAV gene therapy. *Front Immunol*. 2023;14:1227231. doi:10.3389/fimmu.2023.1227231

5. Yang TY, Braun M, Lembke W, et al. Immunogenicity assessment of AAV-based gene therapies: An IQ consortium industry white paper. *Mol Ther Methods Clin Dev*. Sep 8 2022;26:471-494. doi:10.1016/j.omtm.2022.07.018

6. Horton RH, Saade D, Markati T, et al. A systematic review of adeno-associated virus gene therapies in neurology: the need for consistent safety monitoring of a promising treatment. *J Neurol Neurosurg Psychiatry*. Dec 2022;93(12):1276-1288. doi:10.1136/jnnp-2022-329431

7. O'Mahony B, Dunn AL, Leavitt AD, et al. Health-related quality of life following valoctocogene roxaparvovec gene therapy for severe hemophilia A in the phase 3 trial GENEr8-1. *J Thromb Haemost*. Sep 6 2023;doi:10.1016/j.jtha.2023.08.032

8. Ozelo MC, Mahlangu J, Pasi KJ, et al. Valoctocogene Roxaparvovec Gene Therapy for Hemophilia A. *N Engl J Med*. Mar 17 2022;386(11):1013-1025. doi:10.1056/NEJMoa2113708

9. Day JW, Finkel RS, Chiriboga CA, et al. Onasemnogene abeparvovec gene therapy for symptomatic infantile-onset spinal muscular atrophy in patients with two copies of SMN2 (STR1VE): an open-label, single-arm, multicentre, phase 3 trial. *Lancet Neurol*. Apr 2021;20(4):284-293. doi:10.1016/S1474-4422(21)00001-6

10. Ertl HCJ. Immunogenicity and toxicity of AAV gene therapy. *Front Immunol*. 2022;13:975803. doi:10.3389/fimmu.2022.975803

11. Arjomandnejad M, Dasgupta I, Flotte TR, Keeler AM. Immunogenicity of Recombinant Adeno-Associated Virus (AAV) Vectors for Gene Transfer. *BioDrugs*. May 2023;37(3):311-329. doi:10.1007/s40259-023-00585-7

12. Shen W, Liu S, Ou L. rAAV immunogenicity, toxicity, and durability in 255 clinical trials: A meta-analysis. *Front Immunol*. 2022;13:1001263. doi:10.3389/fimmu.2022.1001263

13. Thompson GL, Kavanagh D. Diagnosis and treatment of thrombotic microangiopathy. *Int J Lab Hematol*. Sep 2022;44 Suppl 1(Suppl 1):101-113. doi:10.1111/ijlh.13954

14. Moake JL. Thrombotic microangiopathies. *N Engl J Med*. Aug 22 2002;347(8):589-600. doi:10.1056/NEJMra020528

15. Scully M, Rayment R, Clark A, et al. A British Society for Haematology Guideline: Diagnosis and management of thrombotic thrombocytopenic purpura and thrombotic microangiopathies. *Br J Haematol*. Aug 16 2023;doi:10.1111/bjh.19026

16. Arnold DM, Patriquin CJ, Nazy I. Thrombotic microangiopathies: a general approach to diagnosis and management. *CMAJ*. Jan 30 2017;189(4):E153-E159. doi:10.1503/cmaj.160142

17. Tau J, Fernando LP, Munoz MC, Poh C, Krishnan VV, Dwyre DM. Evaluation of thrombotic thrombocytopenic purpura and other thrombotic microangiopathies: Lessons learned from a 14-year retrospective study. *Ther Apher Dial*. Feb 2023;27(1):136-145. doi:10.1111/1744-9987.13864

18. Martin SD, McGinnis E, Smith TW. Indicators Differentiating Thrombotic Thrombocytopenic Purpura From Other Thrombotic Microangiopathies in a Canadian Apheresis Referral Center. *Am J Clin Pathol*. Nov 8 2021;156(6):1103-1112. doi:10.1093/ajcp/aqab078

19. Rubio-Haro R, Quesada-Carrascosa M, Hernandez-Laforet J, Ferrer Gomez C, De Andres J. Diagnostic-therapeutic algorithm for thrombotic microangiopathy. A report of two cases.

Rev Esp Anestesiol Reanim (Engl Ed). Mar 2022;69(3):179-182. doi:10.1016/j.redare.2020.11.015

20. Abou-Ismail MY, Kapoor S, Citla Sridhar D, Nayak L, Ahuja S. Thrombotic microangiopathies: An illustrated review. *Res Pract Thromb Haemost*. Mar 2022;6(3):e12708. doi:10.1002/rth2.12708

21. Makris M, Pavord S. Most cases of Thrombosis and Thrombocytopenia Syndrome (TTS) post ChAdOx-1 nCov-19 are Vaccine-induced Immune Thrombotic Thrombocytopenia (VITT). *Lancet Reg Health Eur*. Jan 2022;12:100274. doi:10.1016/j.lanepe.2021.100274

22. Schonborn L, Pavord S, Chen VMY, et al. Thrombosis with thrombocytopenia syndrome (TTS) and vaccine-induced immune thrombocytopenia and thrombosis (VITT): Brighton Collaboration case definitions and guidelines for data collection, analysis, and presentation of immunisation safety data. *Vaccine*. Mar 7 2024;42(7):1799-1811. doi:10.1016/j.vaccine.2024.01.045

23. Greinacher A, Thiele T, Warkentin TE, Weisser K, Kyrle PA, Eichinger S. Thrombotic Thrombocytopenia after ChAdOx1 nCov-19 Vaccination. *N Engl J Med*. Jun 3 2021;384(22):2092-2101. doi:10.1056/NEJMoa2104840

24. Schultz NH, Sorvoll IH, Michelsen AE, et al. Thrombosis and Thrombocytopenia after ChAdOx1 nCoV-19 Vaccination. *N Engl J Med*. Jun 3 2021;384(22):2124-2130. doi:10.1056/NEJMoa2104882

25. Pavord S, Scully M, Hunt BJ, et al. Clinical Features of Vaccine-Induced Immune Thrombocytopenia and Thrombosis. *N Engl J Med*. Oct 28 2021;385(18):1680-1689. doi:10.1056/NEJMoa2109908

26. See I, Su JR, Lale A, et al. US Case Reports of Cerebral Venous Sinus Thrombosis With Thrombocytopenia After Ad26.COV2.S Vaccination, March 2 to April 21, 2021. *JAMA*. Jun 22 2021;325(24):2448-2456. doi:10.1001/jama.2021.7517

27. Selvadurai MV, Favaloro EJ, Chen VM. Mechanisms of Thrombosis in Heparin-Induced Thrombocytopenia and Vaccine-Induced Immune Thrombotic Thrombocytopenia. *Semin Thromb Hemost*. Jul 2023;49(5):444-452. doi:10.1055/s-0043-1761269

28. Croskerry P. From mindless to mindful practice--cognitive bias and clinical decision making. *N Engl J Med*. Jun 27 2013;368(26):2445-8. doi:10.1056/NEJMp1303712

29. Webster CS, Taylor S, Weller JM. Cognitive biases in diagnosis and decision making during anaesthesia and intensive care. *BJA Educ*. Nov 2021;21(11):420-425. doi:10.1016/j.bjae.2021.07.004

30. Moake JL, Rudy CK, Troll JH, et al. Unusually large plasma factor VIII: von Willebrand factor multimers in chronic relapsing thrombotic thrombocytopenic purpura. *N Engl J Med*. Dec 2 1982;307(23):1432-5. doi:10.1056/NEJM198212023072306

31. Karmali MA, Petric M, Lim C, Fleming PC, Arbus GS, Lior H. The association between idiopathic hemolytic uremic syndrome and infection by verotoxin-producing Escherichia coli. *J Infect Dis*. May 1985;151(5):775-82. doi:10.1093/infdis/151.5.775

32. Henry N, Mellaza C, Fage N, et al. Retrospective and Systematic Analysis of Causes and Outcomes of Thrombotic Microangiopathies in Routine Clinical Practice: An 11-Year Study. *Front Med (Lausanne)*. 2021;8:566678. doi:10.3389/fmed.2021.566678

33. Chand DH. Clinical findings of thrombotic microangiopathy. Accessed 13 September, 2023. https://www.fda.gov/media/151999/download

34. Al-Nouri ZL, Reese JA, Terrell DR, Vesely SK, George JN. Drug-induced thrombotic microangiopathy: a systematic review of published reports. *Blood*. Jan 22 2015;125(4):616-8. doi:10.1182/blood-2014-11-611335

35. Mazzierli T, Allegretta F, Maffini E, Allinovi M. Drug-induced thrombotic microangiopathy: An updated review of causative drugs, pathophysiology, and management. *Front Pharmacol*. 2022;13:1088031. doi:10.3389/fphar.2022.1088031

36. Bonnemann CG, Belluscio BA, Braun S, Morris C, Singh T, Muntoni F. Dystrophin Immunity after Gene Therapy for Duchenne's Muscular Dystrophy. *N Engl J Med*. Jun 15 2023;388(24):2294-2296. doi:10.1056/NEJMc2212912

37. Guillou J, de Pellegars A, Porcheret F, et al. Fatal thrombotic microangiopathy case following adeno-associated viral SMN gene therapy. *Blood Adv*. Jul 26 2022;6(14):4266-4270. doi:10.1182/bloodadvances.2021006419

38. FDA Cellular T, and Gene Therapies Advisory Committee, #70 CM. Toxicity Risks of Adeno-associated Virus (AAV) Vectors for Gene Therapy (GT). 2021.

39. Chand DH, Zaidman C, Arya K, et al. Thrombotic Microangiopathy Following Onasemnogene Abeparvovec for Spinal Muscular Atrophy: A Case Series. *J Pediatr*. Apr 2021;231:265-268. doi:10.1016/j.jpeds.2020.11.054

40. Salabarria SM, Corti M, Coleman KE, et al. Thrombotic microangiopathy following systemic AAV administration is dependent on anti-capsid antibodies. *J Clin Invest*. Jan 2 2024;134(1)doi:10.1172/JCI173510

41. Zaiss AK, Cotter MJ, White LR, et al. Complement is an essential component of the immune response to adeno-associated virus vectors. *J Virol*. Mar 2008;82(6):2727-40. doi:10.1128/JVI.01990-07

42. Bertin B, Veron P, Leborgne C, et al. Capsid-specific removal of circulating antibodies to adeno-associated virus vectors. *Sci Rep*. Jan 21 2020;10(1):864. doi:10.1038/s41598-020-57893-z

43. Smith CJ, Ross N, Kamal A, et al. Pre-existing humoral immunity and complement pathway contribute to immunogenicity of adeno-associated virus (AAV) vector in human blood. *Front Immunol*. 2022;13:999021. doi:10.3389/fimmu.2022.999021

44. Dunkelberger JR, Song WC. Complement and its role in innate and adaptive immune responses. *Cell Res*. Jan 2010;20(1):34-50. doi:10.1038/cr.2009.139

45. West C, Federspiel JD, Rogers K, et al. Complement Activation by Adeno-Associated Virus-Neutralizing Antibody Complexes. *Hum Gene Ther*. Jun 2023;34(11-12):554-566. doi:10.1089/hum.2023.018

46. Gresele P, Momi S, Marcucci R, Ramundo F, De Stefano V, Tripodi A. Interactions of adenoviruses with platelets and coagulation and the vaccine-induced immune thrombotic thrombocytopenia syndrome. *Haematologica*. Dec 1 2021;106(12):3034-3045. doi:10.3324/haematol.2021.279289

47. Azzarone B, Veneziani I, Moretta L, Maggi E. Pathogenic Mechanisms of Vaccine-Induced Immune Thrombotic Thrombocytopenia in People Receiving Anti-COVID-19 Adenoviral-Based Vaccines: A Proposal. *Front Immunol*. 2021;12:728513. doi:10.3389/fimmu.2021.728513

48. Sun S, Urbanus RT, Ten Cate H, et al. Platelet Activation Mechanisms and Consequences of Immune Thrombocytopenia. *Cells*. Dec 1 2021;10(12)doi:10.3390/cells10123386

49. Marietta M, Coluccio V, Luppi M. Potential mechanisms of vaccine-induced thrombosis. *Eur J Intern Med*. Nov 2022;105:1-7. doi:10.1016/j.ejim.2022.08.002

50. Baglin T. Heparin induced thrombocytopenia thrombosis (HIT/T) syndrome: diagnosis and treatment. *J Clin Pathol*. 2001;54(4):272-4. doi:10.1136/jcp.54.4.272

51. Leung HHL, Perdomo J, Ahmadi Z, et al. NETosis and thrombosis in vaccine-induced immune thrombotic thrombocytopenia. *Nat Commun*. Sep 5 2022;13(1):5206. doi:10.1038/s41467-022-32946-1

52. Roytenberg R, Garcia-Sastre A, Li W. Vaccine-induced immune thrombotic thrombocytopenia: what do we know hitherto? *Front Med (Lausanne)*. 2023;10:1155727. doi:10.3389/fmed.2023.1155727

53. Greinacher A, Warkentin TE. Thrombotic anti-PF4 immune disorders: HIT, VITT, and beyond. *Hematology Am Soc Hematol Educ Program*. Dec 8 2023;2023(1):1-10. doi:10.1182/hematology.2023000503

54. Warkentin TE. Autoimmune Heparin-Induced Thrombocytopenia. *J Clin Med*. Nov 3 2023;12(21)doi:10.3390/jcm12216921

55. Warkentin TE, Arnold DM, Sheppard JI, Moore JC, Kelton JG, Nazy I. Investigation of anti-PF4 versus anti-PF4/heparin reactivity using fluid-phase enzyme immunoassay for 4 anti-PF4 disorders: classic heparin-induced thrombocytopenia (HIT), autoimmune HIT, vaccine-induced immune thrombotic thrombocytopenia, and spontaneous HIT. *J Thromb Haemost*. Aug 2023;21(8):2268-2276. doi:10.1016/j.jtha.2023.04.034

56. Schonborn L, Esteban O, Wesche J, et al. Anti-PF4 immunothrombosis without proximate heparin or adenovirus vector vaccine exposure. *Blood*. Dec 28 2023;142(26):2305-2314. doi:10.1182/blood.2023022136

57. Blachar Y, Leibovitz E, Levin S. The interferon system in two patients with hemolytic uremic syndrome associated with adenovirus infection. *Acta Paediatr Scand*. Jan 1990;79(1):108-9. doi:10.1111/j.1651-2227.1990.tb11340.x

58. Warkentin TE, Baskin-Miller J, Raybould AL, et al. Adenovirus-Associated Thrombocytopenia, Thrombosis, and VITT-like Antibodies. *N Engl J Med*. Aug 10 2023;389(6):574-577. doi:10.1056/NEJMc2307721

59. Campello E, Biolo M, Simioni P. More on Adenovirus-Associated Thrombocytopenia, Thrombosis, and VITT-like Antibodies. *N Engl J Med*. Nov 2 2023;389(18):1729. doi:10.1056/NEJMc2310644

60. Uzun G, Zlamal J, Althaus K, et al. Cerebral venous sinus thrombosis and thrombocytopenia due to heparin-independent anti-PF4 antibodies after adenovirus infection. *Haematologica*. Oct 26 2023;doi:10.3324/haematol.2023.284127

61. Warkentin TE, Greinacher A. Laboratory Testing for Heparin-Induced Thrombocytopenia and Vaccine-Induced Immune Thrombotic Thrombocytopenia Antibodies: A Narrative Review. *Semin Thromb Hemost*. Sep 2023;49(6):621-633. doi:10.1055/s-0042-1758818

62. Petito E, Gresele P. Vaccine-Induced Immune Thrombotic Thrombocytopenia Two Years Later: Should It Still Be on the Scientific Agenda? *Thromb Haemost*. Aug 7 2023;doi:10.1055/a-2107-0891

63. Goad KE, Horne MK, 3rd, Gralnick HR. Pentosan-induced thrombocytopenia: support for an immune complex mechanism. *Br J Haematol*. Dec 1994;88(4):803-8. doi:10.1111/j.1365-2141.1994.tb05120.x

64. Jaax ME, Krauel K, Marschall T, et al. Complex formation with nucleic acids and aptamers alters the antigenic properties of platelet factor 4. *Blood*. Jul 11 2013;122(2):272-81. doi:10.1182/blood-2013-01-478966

65. Sui J, Zheng L, Zheng XL. ADAMTS13 Biomarkers in Management of Immune Thrombotic Thrombocytopenic Purpura. *Arch Pathol Lab Med*. Aug 1 2023;147(8):974-979. doi:10.5858/arpa.2022-0050-RA

66. EMA. Zolgensma (onasemnogene abeparvovec): risk for thrombotic microangiopathy. Accessed 18 September, 2023. https://www.ema.europa.eu/en/documents/dhpc/direct-healthcare-professional-communication-dhpc-zolgensma-onasemnogene-abeparvovec-risk-thrombotic_en.pdf

67. Schulz M, Levy DI, Petropoulos CJ, et al. Binding and neutralizing anti-AAV antibodies: Detection and implications for rAAV-mediated gene therapy. *Mol Ther*. Mar 1 2023;31(3):616-630. doi:10.1016/j.ymthe.2023.01.010

68. Hamilton BA, Wright JF. Challenges Posed by Immune Responses to AAV Vectors: Addressing Root Causes. *Front Immunol*. 2021;12:675897. doi:10.3389/fimmu.2021.675897

Table 1. Comparative characteristics of some TTS

Disease	TMA	Causes	Clinical presentation	Laboratory diagnostic criteria	
TTP	Yes	Failure to degrade ultra-large multimers of von Willebrand factor	● Severe thrombocytopenia (platelet count < 30 x 10^9/l) ● Microangiopathic hemolytic anemia ● New focal neurologic symptoms, seizures, or myocardial infarction (multisystem involvement) ● Kidney injury mild or absent	ADAMTS13 deficiency (< 10% of normal or < 10 IU/dl)	
HUS	Yes	● Exposure to Shiga toxin (classic HUS) ● Defect in complement factors (complement-mediated HUS or atypical HUS)	● Thrombocytopenia ● Microangiopathic hemolytic anemia ● Kidney injury (predominant feature)	● Shiga toxin in stool (classic HUS) ● Genetic testing (complement-mediated HUS)	
TMA associated with AAV-based gene therapy	Yes	Exposure to AAV-vector	● Thrombocytopenia ● Microangiopathic hemolytic anemia ● Kidney injury ● Myocardial inflammation ● Hepatic toxicity	ND	

HIT	No	Heparin treatment	● Thrombocytopenia of moderate severity with onset at 5-14 days after heparin initiation ● Thrombosis	● Normal platelet count before heparin initiation ● Thrombocytopenia defined as a drop in platelet count by 30% to < 100 x 10^9/l or a drop by > 50% from the patient's baseline platelet count ● Positive test for HIT antibodies (including platelet activation test	
VITT	No	Exposure to AV-based anti-SARS-CoV-2 vaccines (leading to anti-PF4 antibodies production)	5-30 days after vaccination: ● New severe headache, not responding to simple analgesia ● Unusual headache that is worse when lying down, or associated with new blurred vision, speech difficulty, motor weakness, drowsiness or seizures ● New unexplained pinprick bruising or bleeding ● Shortness of breath, chest pain, leg swelling, persistent new abdominal pain	● Thrombocytopenia (platelet count < 150 x 10^9/l) ● D-dimer level > 4000 µg/l FEU ● Positive test for antiPF4antibodies (including platelet activation test)	

ADAMTS13, a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13; EIA, enzyme immunoassay; FEU, fibrinogen equivalent unit; HIT, heparin-induced thrombocytopenia; HUS, hemolytic uremic syndrome; ND: not defined; PF4, platelet factor 4; SARS-CoV-2,

severe acute respiratory syndrome coronavirus 2; TM, thrombotic microangiopathy; TTP thrombotic thrombocytopenic purpura; VITT, vaccine-induced immune thrombotic thrombocytopenia.

References: [14, 15, 22, 27, 31,32,44,51]

Emerging thrombotic disorders associated with virus-based innovative therapies: from VITT to AAV-gene therapy-related thrombotic microangiopathy

Silvia Benemei, Francesca Gatto, Rossella Marcucci, Paolo Gresele

Supplementary Table 1. Summary of the TMA-associated AAV gene therapy.

Vector type	**Disease**	**Complement activation**	**Number of patients, age**	**Time of development after gene-therapy administration**	**Outcome**	**Ref**
Onasemnogene abeparvovec	SMA	C5b9 complex increase; C3, C4, FH, FI within normal range	1 female, 6 months	Day 8	Death	Guillou J et al., *Blood Adv*. 2022
Onasemnogene abeparvovec	SMA	C3, C4, Bb fragments, soluble C5b9, CH50, FH autoantibody, FB, FH, FI	3 females, 3-14 months	Day 7	Recovered	Chand DH, et al., *J Pediatr*. 2021
4D-310	Fabry disease	C5b-9 complex increase; Bb fragments increase	1 adult	Day 7	Recovered	Salabarria SM, et al., *J Clin Invest.* 2024
SGT-001, AAV9 vector expressing mini-dystrophin	DMD	widespread complement activation affecting red blood cells	4 males, 7-12 years	Day 6-12	3 recovered, 1 death	Ertl HCJ. *Front Immunol.* 2022; Chand DH *

DMD: Duchenne muscular dystrophy; SMA: spinal muscular atrophy

* https://www.fda.gov/media/151999/download