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**WHOLE EXOME SEQUENCING IN VACCINE-INDUCED
THROMBOTIC THROMBOCYTOPENIA**

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INTRODUCTION

During the COVID-19 pandemic, one of the most critical challenges in managing the global health emergency was the rapid development, testing, and deployment of vaccines against the SARS-CoV-2 virus. These vaccines were introduced in record time, which proved to be instrumental in curbing the spread of the virus, dramatically reducing both the number of deaths and the incidence of severe disease across populations. The various vaccines developed during this period can broadly be categorized into two main types:

mRNA-based vaccines, such as those produced by Moderna and Comirnaty (Pfizer-BioNTech); Adenoviral vector-based vaccines, including Vaxzevria (AstraZeneca) and Ad26.COV2.S (Johnson & Johnson's Janssen vaccine).

While the initial global response to the availability of these vaccines was overwhelmingly positive, a notable shift occurred in February 2021. Around that time, several reports emerged of rare but serious adverse events following vaccination with adenoviral vector vaccines. The first concerns were raised in individuals who had received the Vaxzevria vaccine, followed shortly thereafter - in March 2021 - by similar cases observed in recipients of the Janssen vaccine. These complications were characterized by the simultaneous occurrence of unusual thrombotic events (such as cerebral venous sinus thrombosis or splanchnic vein thrombosis) and thrombocytopenia, occasionally accompanied by bleeding, multiple thromboses, or even disseminated intravascular coagulation (DIC). The striking nature and severity of this condition led several research groups (including Greinacher et al., Pavord et al., and Torjesen I.) to investigate potential underlying mechanisms. This clinical syndrome was soon recognized and defined as Vaccine-Induced Immune Thrombotic Thrombocytopenia (VITT).

The Center for Atherothrombotic Diseases (Department of Experimental and Clinical Medicine, University of Florence/Careggi University Hospital) played a pivotal role in diagnosing and investigating this newly emerging syndrome. Between March and September 2021, the center evaluated more than fifty suspected VITT cases referred by hospitals across Italy. Of those, ten patients were diagnosed with vaccine-associated thrombotic thrombocytopenia.

As part of this PhD project, we aimed to delve deeper into the possible genetic basis of VITT. To achieve this, we leveraged Next Generation Sequencing (NGS) technologies to characterize the genetic and molecular profiles of six of these patients. Specifically, we adopted a Whole Exome Sequencing (WES) approach using the Illumina NextSeq500 platform. This allowed us to investigate molecular pathways that may be closely linked to the development of VITT, providing valuable insights into the biological mechanisms underpinning this rare but serious vaccine-related complication.

VACCINES

The development of a vaccine is typically a lengthy and complex process, often taking 10 to 15 years to complete. Vaccines must undergo multiple preclinical and clinical phases before they can be approved for use. Clinical trials are conducted in three distinct phases, with the final Phase III designed to assess the vaccine's efficacy, meaning its ability to confer protection against the target disease. Each phase is governed by a fundamental requirement: safety. If a vaccine candidate fails to meet safety standards at any stage, the development process is immediately halted. Phase III trials, in particular, are conducted on a large scale, typically involving tens of thousands of participants, in order to detect both rare side effects and to confirm protective efficacy across diverse populations. Once the clinical development is completed successfully, regulatory evaluation is conducted by international and national health authorities. These include the U.S. Food and Drug Administration (FDA), the European Medicines Agency (EMA), and national agencies such as the Italian Medicines Agency (AIFA). Approval processes ensure that the vaccine meets stringent criteria for safety, efficacy, and quality.

It is also important to consider that vaccine development is often slowed down by the high financial risk associated with each phase. The process is resource-intensive, and pharmaceutical companies may proceed cautiously when funding is limited or uncertain. However, in the case of the COVID-19 pandemic, the urgent global need for effective vaccines led to unprecedented levels of public and private investment, as well as international scientific collaboration. This enabled researchers and developers to compress timelines by conducting several phases in parallel, without compromising clinical or safety standards. Despite the accelerated pace, all studies maintained their scientific rigor, and regulatory bodies upheld strict oversight throughout the process. (World Health Organization-WHO)

There are different type of vaccines for the COVID-19 validated by WHO. The first global vaccination program started from December 2020 and the number of administered doses of vaccine is updated daily on the COVID-19 dashboard (WHO).

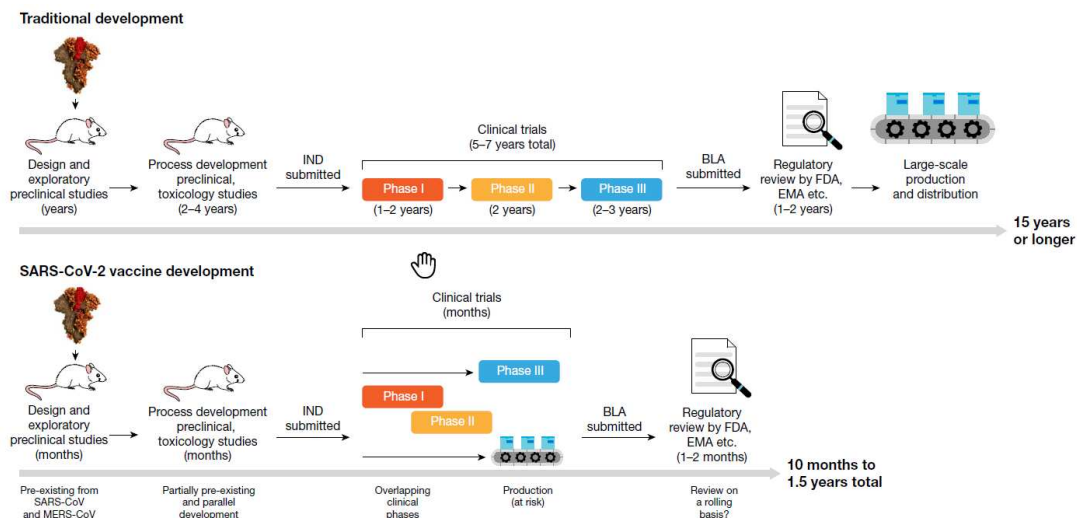


Figure 1: Comparison of traditional and candidate vaccine development against SARS-CoV-2 (source: doi.org/10.1038/s41586-020-2798-3)

The authorized vaccines from EMA are:

- Cominarty
- Spikevax (previously named COVID-19 Moderna)
- Vaxzevria/ChAdOx1 (previously named COVID-19 AstraZeneca)
- Janssen
- Nuvaxovid (Novavax)
- VLA2001 (Valneva)
- Vidprevtyn Beta (Sanofi)

Vaccination Targets

The coronavirus SARS-CoV-2, the causative agent of the disease COVID-19, uses a surface glycoprotein known as the Spike protein (S protein) to bind to and enter human cells, where it subsequently replicates. The vaccines currently available have been designed to elicit an immune response specifically targeted at neutralizing the Spike protein, thereby preventing the virus from gaining entry into host cells. Rather than introducing the whole virus, these vaccines deliver genetic instructions (in the form of mRNA or DNA, depending on the platform) into certain human cells. These instructions temporarily direct the cells to synthesize the Spike protein, which is then displayed on the cell surface or released into the body. The presence of this foreign antigen triggers the immune system to respond by producing neutralizing antibodies that bind to the Spike protein and block viral entry. In addition to the humoral response, the antigen presentation also activates T lymphocytes (T cells). Some of these T cells differentiate into memory T cells, which

can persist in the body for several months or even years. Alongside memory B cells, they form the basis of long-term immunological memory, allowing the immune system to mount a rapid and robust defense upon re-exposure to SARS-CoV-2. (AIFA: Italian Drug Agency)

All currently authorized vaccines for which clinical efficacy data have been published are based on the viral Spike (S) protein as the primary immunogen - either alone or, in the case of inactivated whole-virus vaccines, in combination with other structural viral proteins present in the virion. Due to its critical role in viral entry, specifically in receptor binding and membrane fusion, the Spike protein is the main target of neutralizing antibodies capable of preventing infection. A growing body of evidence supports the conclusion that neutralizing antibodies represent a reliable and robust correlate of protection against SARS-CoV-2 infection (Khoury D. S. et al., 2021 - McMahan K. et al., 2021 - Earle K. A. et al., 2021). During SARS-CoV-2 infection, the Spike (S) protein is synthesized from one of the virus's sub-genomic mRNAs and is co-translationally translocated into the lumen of the endoplasmic reticulum (ER). This process is mediated by an N-terminal signal peptide, spanning amino acid residues 1 to 13 of the full-length protein, which consists of 1,273 amino acids (Zhou P. et al., 2020). The signal peptide is cleaved by a peptidase anchored to the inner membrane of the endoplasmic reticulum (ER), thereby generating the N-terminus of the Spike protein. Following completion of translation, the protein remains anchored to the ER membrane via a C-terminal transmembrane domain, undergoes trimerization, and is then transported to the ER-Golgi intermediate compartment (ERGIC), where viral assembly takes place through budding into the lumen. During exocytosis, the newly formed virions encounter the host protease furin within the trans-Golgi network (TGN). Furin cleaves the Spike protein at a polybasic cleavage site, generating two subunits: the membrane-anchored S2 subunit, and the distal S1 subunit, which remains non-covalently associated until receptor binding (Duan L. et al., 2020; Coutard B. et al., 2020).

The S1 and S2 subunits remain associated as a trimer through non-covalent interactions. Subsequently, the trimer undergoes extensive N-linked and O-linked glycosylation. When the Spike protein is synthesized as a standalone protein - whether via mRNA or adenoviral vectors, or in the context of recombinant subunit vaccine production - the biosynthetic pathway is essentially identical. However, the absence of interaction with other viral structural proteins may influence the glycosylation patterns and/or the stability of the trimeric conformation (Brun J. et al., 2021).

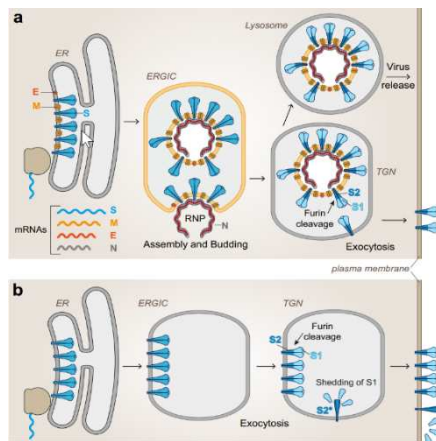


Figure 2: Biosynthesis and Intracellular Trafficking of the Spike Protein.

(a) In infected cells: Subgenomic mRNAs are present and translated to produce structural proteins, with S, M, and E synthesized in association with the endoplasmic reticulum (ER), while N is translated in the cytoplasm. During trafficking, the Spike (S) protein is cleaved into S1 and S2 subunits by furin.

(b) In transfected cells: In the absence of interactions with other viral proteins, post-translational modifications in the trans-Golgi network (TGN) are similar to those in infected cells. However, without stabilizing mutations, the S1 subunit may dissociate ("shedding" phenomenon). (Source: doi.org/10.1038/s41586-020-2798-3)

A number of monoclonal antibodies have been isolated from COVID-19 patients and used to characterize the antigenic features of the trimeric Spike (S) protein, including the determination of 3D structures of complexes formed between the Spike (or specific domains thereof) and the Fab fragments of antibodies. Overall, these studies revealed that the most potent neutralizing antibodies were specific for the receptor-binding domain (RBD). Although neutralizing activity was also observed for antibodies targeting the S2 subunit, their potency was significantly lower compared to those targeting S1 (Andreano E. et al., 2021; Graham C. et al., 2021; Voss et al., 2021). Importantly, the neutralizing antibody response in SARS-CoV-2 infection appears to be dominated by RBD-specific antibodies, which, on average, account for approximately 90% of the total neutralizing activity found in human convalescent sera. Accordingly, a key goal of all COVID-19 vaccines is to present the Spike protein and its RBD in a native-like conformation, in order to induce the production of high levels of potentially neutralizing antibodies following vaccination (Piccoli L. et al., 2020).

Vaccination Types

mRNA vaccines encode a stabilized version of the SARS-CoV-2 Spike protein, which is responsible for viral entry and the associated respiratory syndrome. Clinical trials have demonstrated a 95% efficacy in preventing symptomatic COVID-19 disease. These mRNA-based vaccines elicit a strong humoral immune response, characterized by high titers of neutralizing antibodies, which are considered the primary effectors of protection. Current evidence suggests that circulating antibody levels persist for at least six months following the initial vaccination (first dose), with a modest decline from peak levels observed after the second dose. This antibody waning over time may correlate with an increased incidence of breakthrough infections in the

months following vaccination. Nevertheless, the vaccine-induced immune response remains highly effective at preventing severe disease, hospitalization, and death, even when antibody titers have declined (Goel R.R. et al., 2021).

mRNA vaccines

mRNA vaccines elicit a robust immune response in humans, including the induction of long-lived B and T lymphocytes. Vaccination also promotes the development of Spike-specific CD4⁺ and CD8⁺ T cells. These memory B and T cells can be rapidly reactivated upon re-exposure to the virus, leading to enhanced early control of viral replication and limiting viral dissemination within the host (Goel R.R. et al., 2021).

To enable cellular uptake, mRNA vaccines are formulated as lipid nanoparticle (LNP) complexes, which not only protect the mRNA from enzymatic degradation in tissues but also facilitate its entry into cells and subsequent release into the cytoplasm for translation. The lipid nanoparticle formulation includes a precise mixture of phospholipids, cholesterol, ionizable cationic lipids (which interact with the negatively charged RNA), polyethylene glycol (PEG)-lipids (to stabilize the particles and reduce immunogenicity). These components are combined through a sophisticated and proprietary manufacturing process, for which detailed protocols are not fully disclosed in the scientific literature, to generate the final vaccine product (Verbeke R. et al., 2021). The exact mechanisms by which mRNA is taken up by different cell types, and how it escapes from lipid nanoparticles and intracellular vesicles into the cytoplasm, are not yet fully elucidated (Pardi N. et al., 2017). There are subtle differences between the two major mRNA vaccines - Moderna (mRNA-1273) and BioNTech-Pfizer (BNT162b2) - in terms of mRNA sequence design, LNP composition, and dose per injection. Notably, Moderna's vaccine delivers a higher mRNA dose (100 µg) compared to BioNTech-Pfizer's (30 µg), which may contribute to differences in immunogenicity and durability of the immune response.

Adenoviral Vaccines

The European Medicines Agency (EMA) and the Italian Medicines Agency (AIFA) have so far authorized two viral vector-based COVID-19 vaccines. A viral vector vaccine uses a non-replicating virus, typically an adenovirus, to deliver the genetic sequence of interest into host cells - in this case, the gene encoding the Spike (S) protein of SARS-CoV-2. The immune system is activated in response to the expressed protein and produces protective antibodies, which can neutralize the virus in the event of future exposure, thereby preventing infection. The viral vector vaccines approved by EMA and AIFA are Vaxzevria (AstraZeneca) and the COVID-19 Vaccine Janssen (Johnson & Johnson). The adenoviral vectors used as carriers have been genetically modified to be replication-incompetent, meaning they are unable to reproduce or spread within the human body.

Following vaccination, the adenoviral vectors enter certain human cells, where the DNA fragment that adenoviral vectors carry temporarily initiates production of the SARS-CoV-2 Spike protein. The presence of this foreign antigen triggers the immune system to respond by producing antibodies that specifically bind to the Spike protein, thereby blocking the virus from entering host cells. The foreign Spike protein also activates T lymphocytes, which both assist in antibody production (CD4⁺ helper T cells) and eliminate infected cells (CD8⁺ cytotoxic T cells). After vaccination, some of the antigen-specific lymphocytes persist as memory cells for several months. These memory B and T cells enable the immune system to rapidly mount a strong and coordinated immune response upon any future exposure to SARS-CoV-2. Unlike traditional vaccines, these viral vector vaccines do not contain live or inactivated virus, nor protein subunits, but only a small DNA segment encoding the Spike protein. Expression of the Spike protein is transient, ceasing shortly after antigen presentation has occurred. Currently, four major adenoviral vector-based COVID-19 vaccines are in global use, produced by:

- CanSino Biological Inc./Beijing Institute of Biotechnology, using a human adenovirus type 5 (Ad5) vector (Zhu F. et al., 2020)
- Janssen (Johnson & Johnson), using a human adenovirus type 26 (Ad26) vector (Mercado N.B. et al., 2020; Sadoff J. et al., 2021)
- Oxford-AstraZeneca (ChAdOx1/Vaxzevria), using a chimpanzee adenovirus Y25 vector (Dicks M.D.J. et al., 2012; van Doremalen N. et al., 2020)
- Gamaleya Research Institute (Sputnik V), which uses a heterologous prime-boost strategy: Ad26 for the first dose and Ad5 for the second (Logunov D.Y. et al., 2020; Logunov D.Y. et al., 2021)

A common design feature among all these vaccines is the replacement of the adenoviral early gene E1 with the SARS-CoV-2 S gene, along with deletion of the E3 region. The absence of the E1 gene renders the viral vector replication-deficient. To produce these engineered vaccine particles, immortalized helper cell lines are employed. These cells stably express the E1 gene within their chromosomal DNA, thereby complementing the replication defect. This enables the production of adenoviral structural proteins, replication of the modified viral genome, and ultimately, the assembly of replication-incompetent viral particles used in the vaccine (Hasanpourghadi M., et al. 2021).

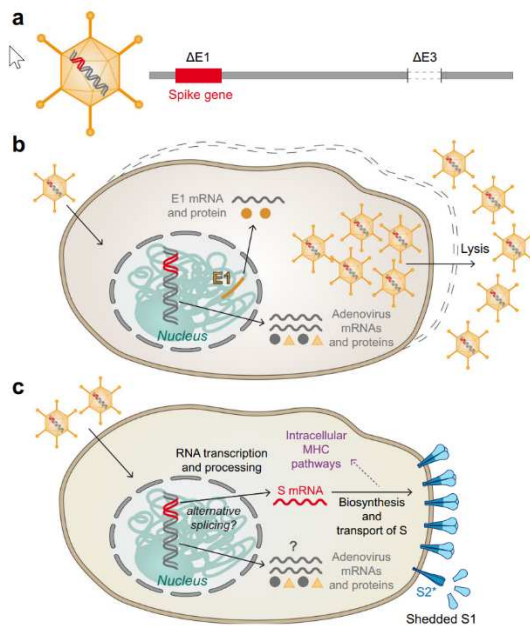


Figure 3: Principles of Adenoviral Vector Vaccines: (a) Structure of the replication-incompetent vector; (b) Formation of vaccine particles in helper cell lines that complement E1; (c) Expression of Spike protein in the cells of vaccinated individuals [source: doi.org/10.1038/s41541-021-00369-6]

The production cell lines used for the Oxford-AstraZeneca, Gamaleya (Sputnik V), and CanSino COVID-19 vaccines are derived from human embryonic kidney (HEK293) cells, while the Janssen (Johnson & Johnson) vaccine is produced using human embryonic retinal cells (PER.C6). Following the amplification and harvesting of adenoviral vector particles, cell lysis is induced using detergents, followed by a series of downstream purification steps to remove host cell components and free viral DNA. Similar to mRNA vaccines, adenoviral vector vaccines are designed to induce the production of native Spike (S) protein via cellular translation of a specific mRNA transcript. However, the pathway leading to this mRNA is significantly more complex than that of direct mRNA vaccines. In this case, the DNA carried by the adenoviral vector must first enter the nucleus of host cells (where it remains episomal, i.e., non-integrating) and undergo transcription and mRNA processing, including splicing, capping, and polyadenylation. Although in vitro studies on the ChAdOx1 nCoV-19 vaccine (Oxford-AstraZeneca) have shown that Spike mRNA transcripts predominate, rare instances of aberrant splicing and non-canonical polyadenylation site usage have been reported (Almuqrin A. et al., 2021). A recent study by Kowarz et al. (2021) provides evidence suggesting that alternative splicing events may lead to the production of C-terminally truncated forms of Spike, potentially resulting in soluble Spike protein variants. The authors hypothesize that these secreted forms could bind to ACE2-expressing endothelial cells and, through antibody-mediated mechanisms, potentially contribute to thrombotic events observed in rare cases following adenoviral vector vaccination (Kowarz E. et al., 2021; Greinacher A. et al., 2021; Hunter P.R., 2021).

Janssen (COVID-19 Ad26.COV2-S vaccine)

Ad26.COV2.S, developed by Janssen Pharmaceutical Companies of Johnson & Johnson, is a recombinant adenovirus type 26 (Ad26) vector-based COVID-19 vaccine. Adenoviruses are a group of viruses that can cause infections in the respiratory and gastrointestinal tracts. The adenovirus used in this vaccine has been genetically modified to be replication-incompetent in human cells and therefore incapable of causing disease. For this vaccine, Janssen employed the same Ad26 vector that was previously used as part of the Ebola vaccine regimen (Ad26.ZEBOV and MVA-BN-Filo). As of December 31, 2020, the Ad26-based platform had been administered to 193,831 participants across various clinical trial programs. These participants included individuals from diverse age groups (infants, children, adults, and the elderly), as well as people living with HIV, pregnant and breastfeeding women. Data from these studies demonstrated a favorable safety profile for the vaccine. The Janssen Ad26-based COVID-19 vaccine elicited a robust humoral immune response, including both neutralizing and non-neutralizing antibody functions, and a strong cellular immune response, involving both CD8⁺ cytotoxic T lymphocytes and CD4⁺ helper T cells - the latter displaying a predominantly Th1 phenotype, irrespective of the transgene-encoded immunogen. This vaccine is monovalent, based on adenovirus serotype 26, and encodes the full-length SARS-CoV-2 Spike (S) protein from the Wuhan-Hu-1 isolate (GenBank accession no. MN908947), stabilized in its prefusion conformation. As described, the vector is replication-incompetent due to deletion of the E1 gene from its genome. To manufacture replication-defective adenoviral vector vaccines, a complementing cell line that provides the missing E1 gene in trans is required. This cell line was derived from a single human retinal cell obtained from fetal tissue in 1985. The line was established via transformation using the adenoviral E1 gene, resulting in a stable cell line that constitutively expresses E1, allowing efficient replication of the Ad26 vector during vaccine production. One advantage of this E1 transformation is that the PER.C6 cell line can be propagated indefinitely, eliminating the need to return to primary fetal tissue at any stage of research or production. The Ad26 vector encoding the SARS-CoV-2 Spike protein is propagated in the PER.C6 TetR cell line, cultured in animal protein-free media supplemented with amino acids. After viral amplification, the vaccine undergoes a series of purification steps, is formulated with inactive ingredients, and filled into vials as the final product (WHO Background Document, 17 March 2021).

ChAdOx1 nCoV-19 (COVID19 AstraZeneca-Vaxzevria vaccine)

ChAdOx1 nCoV-19, developed by the University of Oxford in collaboration with AstraZeneca, was authorized for use by the European Medicines Agency (EMA) on January 29, 2021, and was deployed on a large-scale starting January 1, 2022. Based on data from Phase III clinical trials, the vaccine demonstrated an efficacy of 72% in preventing symptomatic SARS-CoV-2 infection, regardless of the interval between the two doses (data cut-off: January 14, 2021). Notably, higher efficacy was observed in individuals who received two standard doses spaced further apart (up to 12 weeks), suggesting a positive correlation between dosing interval and immune response. The Phase III trial enrolled 32,449 participants, approximately 24% of whom were aged 65 or older. The overall efficacy at the end of the trial was estimated at 76%. No cases of severe COVID-19 were reported in the vaccinated group, while 8 cases occurred in the placebo group. Among participants aged 65 and over, vaccine efficacy was approximately 85%. As the pandemic evolved and new SARS-CoV-2 variants of concern emerged, interim recommendations for vaccine use were updated accordingly. These updates were provided by the World Health Organization (WHO) for the ChAdOx1-S [recombinant] vaccine (also marketed as Vaxzevria™ and COVISHIELD™). Vaxzevria is a non-replicating viral vector vaccine. It uses a genetically modified chimpanzee adenovirus (ChAdOx1) as a delivery vehicle, rendered replication-incompetent and non-pathogenic to humans. The vector carries a DNA sequence encoding the Spike (S) protein of SARS-CoV-2. Once administered intramuscularly, the adenoviral vector enters host cells and delivers its DNA payload into the nucleus. This DNA is then transcribed into mRNA, which exits the nucleus and is translated into the Spike protein by the host cell's ribosomes. The expressed Spike protein, presented on the surface of the host cells, serves as an antigen that triggers the immune response. This immune activation includes both humoral immunity (antibody production by B cells) and cell-mediated immunity (activation of helper and cytotoxic T cells). The vaccine also induces the formation of memory immune cells, which enable a faster and stronger immune response upon future exposure to the virus. The SARS-CoV-2 Spike protein facilitates viral entry into host cells by binding to the angiotensin-converting enzyme 2 (ACE2) receptor, a key molecule involved in blood pressure regulation and tissue protection, especially in the lungs. Once bound, the virus gains entry into the cell, releases its RNA genome, and hijacks the host's cellular machinery to produce viral proteins and assemble new virions, thereby propagating the infection. The vaccine aims to prevent this process by priming the immune system to recognize and neutralize the Spike protein before the virus can enter host cells. As a non-replicating viral vector, Vaxzevria cannot cause infection nor integrate into the human genome. The genetic material coding for the Spike protein is transient and is naturally degraded after initiating the immune response. The adenoviral vector and genetic payload are eliminated

from the body within a few days (Source: Humanitas Research Hospital – IRCCS; WHO interim recommendations on AZD1222).

HIT & VITT

HIT: Heparin-Induced Thrombocytopenia

Heparin-Induced Thrombocytopenia (HIT) is an immune-mediated adverse reaction to heparin, characterized by a decrease in platelet count and a paradoxical increased risk of thrombosis, rather than bleeding.

Historically, two forms of HIT have been distinguished: Type 1 HIT is characterized by a transient, non-immune-mediated mild thrombocytopenia that is usually asymptomatic. In contrast, Type 2 HIT involves an immune-mediated activation that causes and sustains thrombocytopenia associated with platelet activation. Currently, the term HIT is generally reserved for the immune-mediated Type 2 condition. Approximately 1% of patients treated with heparin develop HIT, and about 50% of these patients experience thrombotic complications. The disorder has a slightly higher prevalence in females (Orphanet).

Heparin-induced thrombocytopenia is a prothrombotic, autoimmune, and potentially life-threatening complication of heparin therapy. The pathogenic mechanism involves antibody-mediated platelet activation and consequent thrombin generation, leading to a paradoxical clinical scenario: despite heparin being an anticoagulant, HIT primarily manifests as an increased risk of venous and/or arterial thrombosis (Greinacher A., 2009; Greinacher et al., 2017). Early diagnosis and initiation of non-heparin anticoagulants are crucial to prevent further complications. Diagnosis can be challenging, especially in patient populations with a high baseline incidence of thrombocytopenia. Diagnostic assays for HIT antibodies exhibit high negative predictive value but relatively low positive predictive value; therefore, test results must be interpreted in the clinical context. Optimal diagnosis requires integrating both the clinical presentation and detection of pathogenic HIT antibodies, emphasizing the importance of clinical and laboratory correlation (Greinacher et al., 2009; Greinacher et al., 2017). HIT can occur at any age over three months, although pediatric cases are rare. Moderate thrombocytopenia typically develops 5 to 10 days after heparin initiation. In patients previously exposed to heparin within the last 100 days, rapid onset can occur with platelet count drops within minutes to hours after re-exposure. Delayed onset HIT has also been reported, with thrombocytopenia developing after heparin discontinuation. Thrombocytopenia is usually asymptomatic, and bleeding is rare. However, HIT is associated with a high risk of thrombotic complications, including pulmonary embolism, myocardial infarction, and thrombotic stroke. Thrombosis frequently involves arterial vessels of the limbs and deep venous thrombosis. Microvascular thrombosis can lead to venous limb gangrene, which may necessitate amputation. Other complications include heparin-induced skin necrosis at injection sites and anaphylactoid reactions (fever, hypotension, chest pain, dyspnea, cardiopulmonary arrest) after intravenous bolus administration of heparin (Orphanet).

HIT results from the formation of immune complexes composed of autoantibodies targeting the platelet factor 4 (PF4)–heparin complex, which bind to platelet and monocyte surfaces. These autoantibodies specifically recognize PF4 only when complexed with heparin. The immune complex triggers activation of circulating platelets via their FcγRIIA receptors, leading to platelet consumption and a hypercoagulable state. Once antibodies are formed, a subgroup of patients develops a profound hypercoagulable state caused by cell-activating antibodies that promote thrombin generation (Orphanet).

HIT is an immune-mediated autoimmune disorder:

Immune-mediated because the IgG antibodies are directed against neoepitopes formed on PF4 after its physiological binding to heparin on the platelet surface.

Autoimmune because the IgG do not target the drug itself but recognize endogenous PF4 protein regions.

The antibodies stimulate tissue factor expression and their Fc portions bind FcγRIIa receptors on platelets and monocytes, resulting in activation of platelets, endothelial cells, and monocytes. This activation causes platelet degranulation with recruitment of additional platelets, increased endothelial adhesiveness, and phagocytosis.

It is important to note that anti-PF4 antibodies are quite common in heparin-treated patients (almost 50%), but most do not cause platelet aggregation. Therefore, diagnosis must rely on clinical presentation, with antibody testing serving as a confirmatory tool. Anti-PF4/heparin antibodies are extremely rare in healthy individuals (prevalence 0.3–0.5%). Furthermore, infection and surgical inflammation may trigger spontaneous HIT, a rare variant occurring without prior heparin exposure. In such cases, unexplained thrombocytopenia and/or thrombosis are associated with laboratory evidence of high-titer platelet-activating anti-PF4/heparin antibodies (Khandelwal & Arepally, 2016).

Clinical Features and Thrombotic Complications of HIT

HIT typically presents with a greater than 50% drop in platelet count (from the highest value after day 4 of heparin exposure) and is frequently associated with new thrombotic events, most often occurring 5-14 days after the initiation of prophylactic or therapeutic heparin dosing. Thromboembolic complications primarily affect the venous system. Rare complications include skin necrosis, adrenal hemorrhagic necrosis, or anaphylactic reactions following intravenous heparin bolus administration (Greinacher, 2009; Greinacher et al., 2017; Greinacher et al., 2021). The more unusual a new thrombotic event appears during heparin treatment; the more HIT should be suspected. HIT-associated thromboses often coincide with a ~50% platelet drop, but can occasionally precede thrombocytopenia by 1-2 days. In cases of re-exposure to heparin within 30 days, HIT may present as rapid-onset HIT, where thrombocytopenia and thrombosis develop within the first 24 hours of heparin administration due to pre-existing circulating anti-PF4

antibodies. Delayed-onset HIT, occurring several days to weeks after discontinuation of heparin, is caused by platelet-activating antibodies that act independently of heparin, mimicking an autoimmune-like syndrome.

In extremely rare cases, spontaneous HIT occurs without prior heparin exposure, caused by endogenous anti-PF4 IgG antibodies with the same pathogenic properties as those seen in classical HIT.

Thrombocytopenia in HIT develops due to antibody-mediated platelet aggregation, and may occur:

- Within 3 days of heparin initiation if anti-PF4 antibodies from a previous exposure (within the past 150 days) are still present;
- Between 4 to 10 days, which is typical for the development of a new immune response;
- Less commonly, after 10 days.

Cases of spontaneous autoimmune HIT (aHIT) have been described in the literature. These patients have never received heparin yet harbor platelet-activating anti-PF4 IgG that induce massive, uncontrolled, and widespread thrombosis (HIT + T). This severe thrombotic syndrome is observed in 35-75% of HIT patients. Bleeding is rare, and when it does occur, it classically involves adrenal arteries. This is a list of most frequent thrombotic complications associated with HIT:

- Deep vein thrombosis (DVT) and pulmonary embolism (PE)
- Ischemic stroke
- Critical limb ischemia with gangrene, often leading to limb amputation
- Acute myocardial infarction
- Renal infarction with acute kidney injury
- Cerebral venous sinus thrombosis (CVST)
- Retinal artery thrombosis, leading to anopsia or hemianopsia

Pathogenesis

Circulating heparin molecules bind to platelet factor 4 (PF4), a positively charged plasma protein released during platelet activation. Under physiological conditions, PF4 interacts with negatively charged glycosaminoglycans on the endothelial surface, displacing antithrombin and thereby promoting coagulation. However, PF4 exhibits higher affinity for heparin, forming PF4–heparin complexes that act as neoantigens, triggering an immune response and the production of anti-PF4/heparin IgG antibodies. These immune complexes (PF4–heparin–IgG) bind to FcγRIIA receptors on the platelet surface, resulting in platelet activation, aggregation, and the release of additional PF4, thus initiating a positive feedback loop that sustains and amplifies platelet activation. In parallel, these immune complexes also activate monocytes, which release tissue

factor, further amplifying the coagulation cascade and contributing to a hypercoagulable state. Once initiated, this reaction becomes self-perpetuating, driving ongoing platelet consumption and thrombin generation (Marcucci & Marietta M., 2021).

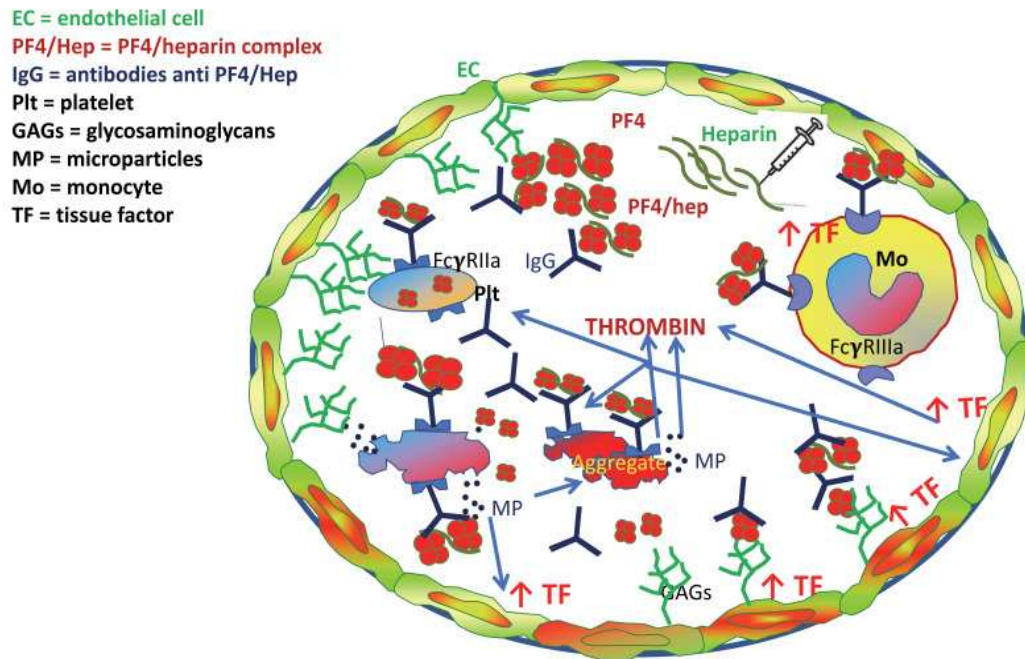


Figure 4: Circulating heparin molecules bind to platelet factor 4 (PF4), a positively charged plasma protein released during platelet activation. The resulting heparin–PF4 complexes act as neoantigens, triggering the formation of antibodies directed against the complex itself. These heparin–PF4–IgG immune complexes engage FcγRIIA receptors on the surface of platelets, leading to their activation, aggregation, and the release of additional PF4, thereby establishing a positive feedback loop that further amplifies platelet activation. In addition, these immune complexes activate monocytes, which release tissue factor, promoting concurrent activation of the coagulation cascade and contributing to a prothrombotic state (Marcucci R. & Marietta M., 2021).

HIT antigens are frequently found on platelet factor 4 (PF4) when complexed with heparin or other sulfated polysaccharides, forming linear multimolecular clusters. The size, quantity, and stability of these PF4/heparin complexes critically influence their immunogenic potential. Anti-PF4/heparin antibodies bind to PF4 via their F(ab) region. When PF4 molecules are arranged in a linear, repetitive fashion, multiple IgG antibodies - the immunoglobulin isotype primarily involved in HIT - can bind adjacently, promoting the formation of large immune complexes. These complexes crosslink and activate platelet FcγRIIA receptors, triggering platelet activation, aggregation, and a procoagulant response. Importantly, thrombocytopenia in HIT is not due to reticuloendothelial phagocytosis, but rather to intravascular platelet activation and the subsequent release of procoagulant platelet-derived microparticles. The severity of thrombocytopenia correlates with thrombotic risk. Antibodies targeting other heparin-binding proteins (e.g., interleukin-8, neutrophil-activating peptide-2) play a minor role in the pathogenesis of HIT. Moreover, HIT antibodies can bind to endothelial cells, inducing procoagulant changes in microvascular endothelial cells. These antibodies also activate monocytes and neutrophils,

leading to the formation of platelet–monocyte–neutrophil aggregates. This pan-cellular activation may contribute significantly to the prothrombotic nature of HIT (Greinacher et al., 2009; Greinacher et al., 2017).

Diagnosis and Laboratory Testing of Heparin-Induced Thrombocytopenia (HIT)

The diagnosis of HIT involves a two-step approach:

- Clinical Assessment: Utilizing the 4T score, a validated tool designed to estimate the probability of HIT occurrence.
- Detection of Antibodies Binding to the Heparin/PF4 Complex: Identifying antibodies that interact with the heparin/PF4 complex is crucial for confirming the diagnosis.

4T Score

The 4T score is a clinical scoring system based on available patient characteristics during heparin therapy. It serves as a preliminary diagnostic tool while awaiting laboratory results. The score considers four elements

- Thrombocytopenia: Assessment of platelet count reduction.
- Timing of Platelet Count Fall: When the platelet count decreases in relation to heparin exposure.
- Thrombosis: Presence of new or progressive thrombotic events.
- Other Causes of Thrombocytopenia

The resulting score categorizes the probability of developing HIT as low, intermediate, or high. In cases of intermediate or high probability, further clinical analyses are recommended, such as testing for anti-PF4/heparin antibodies and discontinuing heparin administration pending additional clinical evaluations. It is important to note that the 4T score does not replace laboratory tests for HIT diagnosis; results should be interpreted within the clinical context. Additionally, the 4T score may be less effective in intensive care unit settings. Alternative scoring systems, like the HIT Expert Probability (HEP) score, have been developed to enhance performance in specific patient populations. However, due to its complexity, the 4T score remains the most widely used approach for general HIT diagnosis (Marcucci R. & Marietta M., 2021).

Currently, two main types of laboratory tests are employed for HIT diagnosis (Marcucci R. & Marietta M., 2021):

- Immunological Assays: These tests determine and quantify the presence of anti-PF4/heparin antibodies;
- Functional Assays: These assess the ability of anti-PF4/heparin antibodies to bind and activate platelets.

Immunological Assays

Enzyme-Linked Immunosorbent Assay (ELISA): This test involves the target antigen (PF4/polyanion complex) being immobilized on a solid phase, followed by the addition of patient plasma or serum. An enzymatic reaction produces a photometric change indicating the presence of HIT antibodies. The intensity of this change correlates with antibody concentration and is reported in optical density (OD). Various commercial ELISA kits are available, differing primarily in the type of antigen used. Recent developments include assays like PaGIA, based on the agglutination of polystyrene particles coated with PF4/heparin complexes.

Chemiluminescence Immunoassays (CLIA): These assays detect anti-PF4/heparin antibodies by measuring the light emitted during a chemical reaction. Higher positivity levels increase the likelihood of confirming HIT with subsequent functional tests. Some CLIA-based tests are fully automated, providing results within an hour.

While ELISA and CLIA tests are widely available and offer high sensitivity, they have limitations in specificity. Not all detected antibodies are capable of activating platelets, which can lead to false positives. Therefore, these tests are often used as screening tools, with positive results requiring confirmation through functional assays.

Functional Assays

Serotonin Release Assay (SRA): Considered the gold standard for HIT diagnosis, the SRA measures the release of radiolabeled serotonin from donor platelets upon exposure to patient plasma containing anti-PF4/heparin antibodies. A positive result is characterized by serotonin release at pharmacological heparin concentrations, which is inhibited at higher heparin levels. Despite its high sensitivity and specificity, the SRA is technically demanding, involves radioactive materials, and is not widely available.

Heparin-Induced Platelet Activation Test (HIPA): This test evaluates platelet activation in the presence of patient plasma and heparin. It is considered a reliable alternative to the SRA, especially in Europe. The HIPA test is more accessible but still requires specialized laboratory facilities and expertise.

Functional assays are more specific for HIT and provide a higher positive predictive value compared to immunological assays. However, they are less sensitive and more complex to perform. Therefore, a combination of initial immunological screening followed by functional testing is recommended for accurate HIT diagnosis. [ncbi.nlm.nih.gov](https://www.ncbi.nlm.nih.gov)

In summary, the diagnosis of HIT requires a combination of clinical evaluation and laboratory testing. The 4T score serves as an initial assessment tool, while immunological assays screen for the presence of anti-PF4/heparin antibodies. Functional assays confirm the pathogenicity of these antibodies. A structured diagnostic approach ensures accurate and efficient identification of HIT, enabling prompt and effective treatment (Marcucci R. & Marietta M., 2021).

Treatment of HIT (Heparin-Induced Thrombocytopenia)

Treatment involves two main strategies:

- Immediate discontinuation of all forms of heparin exposure: this step is essential but not sufficient on its own. As previously described, once initiated, the process of platelet and coagulation activation becomes self-perpetuating.
- Initiation of a therapeutic-dose anticoagulant that is not heparin-based.

Stopping heparin alone does not adequately prevent thrombotic complications. Thrombotic events still occur in 19% to 52% of patients within the first month after discontinuation. Therefore, when HIT is strongly suspected, alternative anticoagulation should be initiated as soon as possible (Marcucci R. & Marietta M., 2021).

The non-heparin anticoagulants currently available for the treatment of HIT include:

- Argatroban;
- Bivalirudin;
- Danaparoid;
- Fondaparinux;
- Direct Oral Anticoagulants (DOACs);
- Lepirudin, which was withdrawn from the market in 2012.

Each of these agents offers different pharmacological profiles and is selected based on clinical context, including renal or hepatic function, bleeding risk, and the need for surgical or periprocedural anticoagulation.

Immune Response in HIT

One of the most intriguing aspects of Heparin-Induced Thrombocytopenia (HIT) is its underlying immunobiology (Greinacher et al., 2009; Greinacher et al., 2017; Greinacher et al., 2021). Uniquely, HIT does not exhibit the typical characteristics of either a primary immune response - namely, an initial production of IgM antibodies followed by a delayed IgG response - nor those of a classical secondary immune response, which is marked by a stronger and more sustained IgG response. Instead, most patients with HIT develop high levels of IgG antibodies between days 4 and 10 after heparin exposure. This pattern more closely resembles a T-cell-independent B-cell response, similar to that observed in immune reactions against viral antigens displaying repetitive epitopes (Zinkernagel RM & Hengartner H., 2001). In fact, in HIT, the repetitive epitopes are expressed as highly ordered, ridge-like linear clusters of PF4/heparin complexes measuring approximately 100–150 nm in size, with inter-epitope distances of 4–6 nm (Greinacher et al.,

2009; Greinacher et al., 2017). These structural features resemble those known to activate B cells in a T-cell-independent manner. However, other studies point toward the involvement of T-cell-dependent mechanisms in HIT pathogenesis. T-cell-independent B-cell responses typically lead to IgM production, yet IgG antibodies predominate in HIT. In a murine model, the immune response against PF4/heparin complexes was found to be T-cell-dependent (Suvana S. et al., 2005), suggesting prior immunological sensitization to HIT antigens. According to Greinacher et al. (2009), early exposure to PF4-containing complexes - possibly triggered by endogenous, non-heparin polyanions - could lead to PF4 clustering and initiate a T-cell-dependent class switch in B cells. Later, these memory B cells - most likely marginal zone B cells (Allman D. & Pillai S., 2008) - may be reactivated in a transient manner when PF4 clusters form upon heparin administration, especially in a pro-inflammatory context. A deeper understanding of HIT immunobiology may offer valuable insights into how the host immune system, under certain conditions, transitions from defense to autoimmune pathology.

Autoimmune HIT (aHIT) as a Model for VITT

A distinct clinical syndrome not triggered by heparin exposure has been identified and termed autoimmune HIT (aHIT) (Greinacher et al., 2009; Greinacher et al., 2017). The hallmark of this condition is the presence of circulating antibodies capable of activating platelets in the absence of heparin. Polyanionic molecules potentially implicated in the development of aHIT include bacterial and viral components, highly sulfated chondroitin sulfate, DNA, RNA, and polyphosphates (Warkentin T.E. & Greinacher A., 2021). Patients with autoimmune HIT typically present with clinical features that differ from classic HIT, including severe thrombocytopenia ($<20 \times 10^9/L$), which is sometimes accompanied by disseminated intravascular coagulation (DIC), microvascular thrombosis, and cerebral venous sinus thrombosis (CVST) in up to 40% of cases (Warkentin T.E. & Greinacher A., 2021). Shortly after reports emerged of unusual thrombotic events associated with the Vaxzevria (AstraZeneca) COVID-19 vaccine, a German research group proposed a hypothetical pathogenic mechanism underlying these rare events, naming it Vaccine-Induced Prothrombotic Immune Thrombocytopenia (VIPIT) - now widely known as VITT - based on findings from nine vaccinated individuals (Greinacher et al., 2009; Greinacher et al., 2017). In the sera of four of these patients, anti-PF4/heparin antibodies were detected using an enzyme-linked immunosorbent assay (ELISA). These antibodies were inhibited by high concentrations of heparin (100 U/mL), and the sera could activate control washed platelets in vitro when either PF4 or the Vaxzevria vaccine was added. The study also demonstrated that platelet activation was FcγRIIA-dependent, as it was blocked by a specific FcγRIIA antagonist. Given the absence of prior heparin exposure, the authors proposed that VITT

represents an autoimmune HIT-like syndrome (Gresele P. et al., 2021).

In a separate study, McGonagle et al. suggested that microtrauma from vaccine injection may bring adenoviral DNA into contact with PF4, thereby promoting the production of anti-PF4 autoantibodies in genetically or immunologically susceptible individuals (McGonagle D. et al., 2021). High-titer circulating anti-PF4 autoantibodies may then drive immunothrombosis in anatomically susceptible vascular beds, such as the portal or cerebral venous systems, where PF4 is known to interact intensively with microbial and viral components to maintain immune surveillance and antigen clearance.

Another proposed mechanism involves the generation of soluble spike protein variants through alternative mRNA splicing, which may bind to ACE2 receptors on endothelial cells, triggering antibody-dependent cellular cytotoxicity (ADCC) or complement-dependent cytotoxicity (CDC) (Kowarz E. et al., 2022). Understanding autoimmune HIT has been instrumental in framing VITT as a novel vaccine-related immune thrombotic disorder, further bridging the fields of immunohematology and vaccinology.

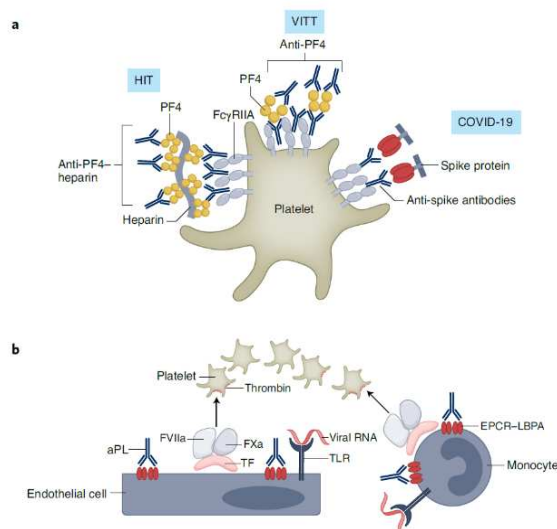


Figure 5: Platelet activation in HIT, VITT, and COVID-19: (a) Antibodies against PF4 in VITT and HIT, as well as antibodies against the SARS-CoV-2 spike protein in COVID-19 patients, activate platelets through FcγRIIA signaling triggered by IgG aggregated within immune complexes; (b) Direct platelet activation via FcγRIIA can be amplified in severe COVID-19 by coagulation activation through convergent activity of pathogen recognition receptors and antiphospholipid antibodies (aPL) on monocytes or endothelial cells. [Source: 10.1038/s41590-021-01042-9]

The antibodies involved in HIT target PF4 bound to negatively charged heparin polymers, forming large pathological immune complexes that activate platelets via the FcγRIIA (CD32a) receptor (Arman M. & Krauel K. J., 2015). In contrast, antibodies in autoimmune HIT cluster PF4 tetramers, thereby activating platelets in the absence of heparin. Consequently, a variety of factors influencing the size and stability of anti-PF4 immune complexes may explain both the pathogenesis and phenotypic diversity of HIT. Of particular importance is the binding affinity of anti-PF4 antibodies; however, equally relevant are the electrostatic interactions between the

polycationic PF4 and polyanions. Besides exogenous heparin, these polyanions could include glycosaminoglycans and polyphosphates associated with platelets or other vascular and tissue cells. Therefore, organ-specific characteristics and individual platelet phenotypes can affect the assembly, size, and physicochemical properties of complexes formed between anti-PF4 antibodies, PF4 itself, and anionic species. Activation of FcγRIIA leads to increased procoagulant activity, resulting in enhanced thrombin generation, fibrin deposition, and thrombin-mediated feedback regulation of platelet activation. This establishes a self-sustaining prothrombotic environment within the vessel lumen and on cellular surfaces. The end result is a thrombocytopenic syndrome caused by platelet consumption and immunothrombosis, with IgG anti-PF4 antibodies present in both HIT and VITT. Anti-PF4 antibodies in VITT and HIT - whether heparin-dependent or independent - exhibit similar binding characteristics, suggesting comparable capacities to form platelet-activating immune complexes (Ruggeri Z.M. & Wolfram R., 2021).

More recently, the German research group proposed that the adenoviral vector and/or certain protein components of the Vaxzevria vaccine activate platelets, causing PF4 release. PF4 then forms complexes with viral proteins and other anionic vaccine constituents, generating neoantigens that elicit antibodies inducing strong platelet activation via FcγRIIA. This stimulates granulocyte activation and ultimately leads to catastrophic thrombosis (Greinacher A. et al., 2009; 2017; 2021). Furthermore, the presence of EDTA in the vaccine may promote vascular leakage at the injection site, facilitating systemic dissemination of vaccine components.

In a recent mutagenesis study (Huynh A. et al., 2021), the binding sites on PF4 of antibodies isolated from VITT and classic HIT patients were explored. VITT anti-PF4 antibodies bound to eight surface amino acids located exclusively within the PF4 heparin-binding site, whereas HIT anti-PF4 antibodies bound amino acids corresponding to two distinct sites on PF4. Additionally, VITT antibodies exhibited stronger binding responses than HIT antibodies. The authors concluded that VITT antibodies mimic heparin's effect by binding to a similar site on PF4, enabling PF4 tetramer clustering and immune complex formation, which in turn triggers FcγRIIA-dependent platelet activation (Huynh A. et al., 2021).

These unique features explain why VITT requires different testing approaches compared to classic HIT. In suspected VITT cases, anti-PF4/heparin antibodies are detected by ELISA but often not by rapid assays typically used for HIT screening (Platton S. et al., 2021).

While the autoimmune HIT hypothesis is important and provides a foundation for understanding this new catastrophic autoimmune thrombotic syndrome, several aspects do not fully align with the clinical presentation of VITT, and many questions remain unresolved.

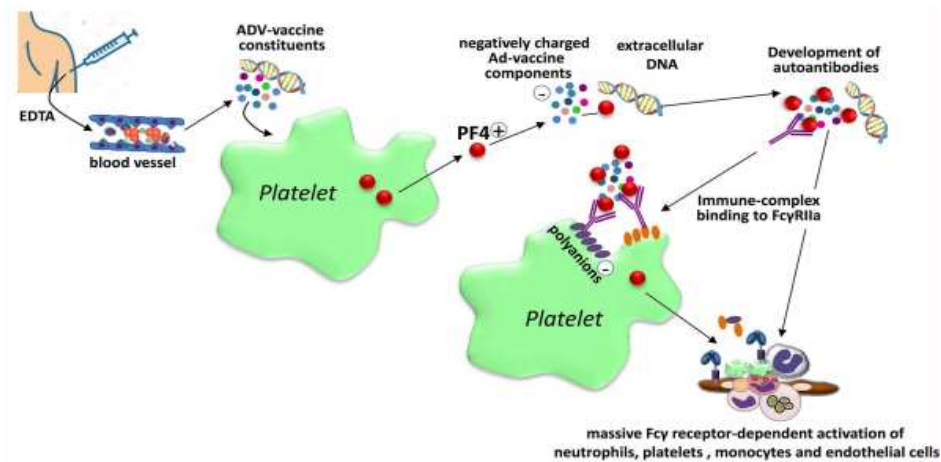


Figure 6: Hypothesis of vaccine-induced autoimmune thrombocytopenia and similarities with VITT: vaccine components that leak into the bloodstream from the injection site (facilitated by ethylenediaminetetraacetic acid, EDTA, present in the vaccine) activate platelets to release platelet factor 4 (PF4). Vaccine constituents, likely polyanions or viral DNA, form complexes with positively charged PF4 that are recognized as neoantigens by B cells, which then produce antibodies against these complexes. The resulting immune complexes activate platelets via FcγRIIa, triggering the release of additional PF4 and polyphosphates, thereby initiating a positive feedback loop leading to further platelet activation and consumption. Extracellular DNA within neutrophil extracellular traps binds PF4, and the resulting DNA/PF4 complexes further recruit anti-PF4 antibodies, inducing massive Fc receptor-dependent activation of neutrophils, platelets, monocytes, and endothelial cells, culminating in extensive coagulation activation and thrombosis (Gresele P. et al., 2021)

VITT (Vaccine-induced Immune Thrombotic Thrombocytopenia)

The widespread availability of COVID-19 vaccines significantly altered the trajectory of the pandemic. Mass vaccination campaigns across the globe helped reduce the number of deaths and severe disease, particularly in countries that implemented large-scale immunization programs (Greinacher A. et al., 2009; 2017; 2021). To date, the following COVID-19 vaccines have been authorized by the World Health Organization (WHO) for emergency use. Regulatory approvals vary across agencies such as the FDA, EMA, and national drug authorities. These include:

- Comirnaty – Pfizer/BioNTech (mRNA)
- Spikevax – Moderna (mRNA)
- Vaxzevria – AstraZeneca (adenoviral vector)
- Jcovden – Johnson & Johnson (adenoviral vector)
- CoronaVac – Sinovac (inactivated virus)
- Covilo – Sinopharm (inactivated virus)
- Covaxin – Bharat Biotech (inactivated virus)
- Nuvaxovid – Novavax (recombinant protein)
- Convidecia – CanSino (adenoviral vector)
- Valneva – Valneva (inactivated virus)

Initial enthusiasm was tempered in February 2021, when reports emerged of rare but serious thrombotic events associated with thrombocytopenia following administration of ChAdOx1 nCoV-19 (Vaxzevria) and Ad26.COV2.S (Janssen/Johnson & Johnson) vaccines (Marcucci R. & Marietta M., 2021). As previously mentioned, both vaccines use adenoviral vectors to deliver the mRNA encoding the SARS-CoV-2 spike protein into host cells.

A pharmacovigilance review conducted through EudraVigilance, the European database of suspected adverse drug reaction reports, highlighted a safety signal of unusual thromboembolic events - including cerebral venous sinus thrombosis (CVST), splanchnic vein thrombosis (SVT), and arterial thrombosis (AT) - with or without thrombocytopenia, occurring predominantly in women under 60 years of age within 14 days post-vaccination (Marcucci R. & Marietta M., 2021). On April 7, 2021, the Pharmacovigilance Risk Assessment Committee (PRAC) of the European Medicines Agency (EMA) concluded that a causal relationship between Vaxzevria and these rare events was at least a reasonable possibility. However, it reaffirmed that the overall benefit of Vaxzevria in preventing COVID-19 outweighed the risks, including the risk of thrombosis with thrombocytopenia syndrome (TTS) (EMA, 2021).

Following this, European countries resumed vaccination with certain restrictions. For instance, in Germany, Italy, and Spain, Vaxzevria was recommended only for individuals over 60 years old, in the UK, for those over 30 and in France, for those over 55.

As of the latest data for AstraZeneca (ChAdOx1 nCoV-19) 9.4 VITT cases per million doses in the UK (309 cases out of 32.9 million doses) and 4 cases per million doses in Spain, with a higher rate (14–15 per million) among those aged 30–49 are reported. While for Johnson & Johnson (Ad26.COV2.S) 3.2 cases per million doses in the U.S. (28 cases out of 8.7 million doses) and 1 case per 500,000 doses in Ontario, Canada are reported.

While VITT remains extremely rare, its severity prompted several countries to suspend or restrict the use of adenoviral vector vaccines, especially in certain age groups. It is crucial that healthcare professionals remain vigilant for signs of VITT and that patients are informed of potential risks.

After a thorough safety review, the U.S. Food and Drug Administration (FDA), the Centers for Disease Control and Prevention (CDC), and the EMA's PRAC determined that vaccination with Ad26.COV2.S could safely resume. As a precaution, the Italian Medicines Agency (AIFA) recommended preferential use of this vaccine in individuals aged 60 and older (FDA News).

This newly recognized clinical entity, characterized by cerebral or splanchnic vein thrombosis, often in association with multiple thrombotic events, thrombocytopenia, hemorrhages, and in some cases disseminated intravascular coagulation (DIC), has been classified as a distinct syndrome known as VITT (*Vaccine-Induced Immune Thrombotic Thrombocytopenia*) (Greinacher A. et al., 2009; 2017; 2021). The condition has been observed predominantly in women under the age of 55, typically occurring between 4- and 16-days following administration of adenoviral vector-based COVID-19 vaccines. These observations led regulatory authorities in several countries to enhance pharmacovigilance efforts. Initially, vaccinations with these products were suspended, and later their use was restricted to specific age groups (Marcucci R. & Marietta M., 2021). According to the American Society of Hematology (ASH), VITT is defined as a clinical syndrome characterized by the presence of all of the following radiological and laboratory abnormalities in individuals vaccinated with an adenoviral vector COVID-19 vaccine (Ad26.COV2.S or ChAdOx1 nCoV-19), with symptom onset between 4- and 30-days post-vaccination:

- Thrombosis in atypical locations, particularly cerebral venous sinus thrombosis (CVST) and splanchnic vein thrombosis;
- Moderate to severe thrombocytopenia. However, a normal platelet count at presentation should not rule out early-stage VITT;
- Presence of anti-platelet factor 4 (PF4) antibodies, as detected by a positive enzyme-linked immunosorbent assay (ELISA) (Aleem A. & Nadeem AJ, 2021).

Epidemiology of VITT

This syndrome has been referred to by several names, but the most widely adopted term is Vaccine-Induced Immune Thrombotic Thrombocytopenia (VITT). The name reflects the syndrome's pathophysiological resemblance to heparin-induced thrombocytopenia (HIT) - despite VITT occurring in the absence of heparin exposure. The clinical and laboratory similarities to HIT are supported by the detection of high-titer anti-platelet factor 4 (PF4) antibodies capable of activating platelets in patient serum.

VITT - also referred to as Thrombosis with Thrombocytopenia Syndrome (TTS) - has been primarily observed in women under the age of 55, occurring between 4 and 16 days after administration of the Vaxzevria vaccine (ChAdOx1 nCoV-19, AstraZeneca) or, at an even higher incidence, after the Janssen (Ad26.COV2.S) vaccine. The condition has been associated with a notably high mortality rate (See I. et al., 2021). Although COVID-19 vaccines were rigorously tested in large clinical trials involving thousands of participants before receiving regulatory approval, even the largest trials are underpowered to detect extremely rare adverse events, such as those occurring at rates below 1 in 100,000. Therefore, the identification of such events in post-marketing pharmacovigilance reports is not unexpected - especially given the vast number of vaccinated individuals worldwide, particularly in Western countries.

In March 2021, concerns began to surface regarding an increased risk of thrombosis associated with thrombocytopenia among individuals who had received ChAdOx1 nCoV-19. By the end of that month, healthcare providers in Norway, Germany, Austria, and the United Kingdom reported hospitalizations occurring 5 to 24 days post-vaccination. These patients presented with unusual site thromboses, thrombocytopenia, markedly elevated D-dimer levels, and reduced fibrinogen concentrations. Most of the affected individuals were previously healthy and relatively young (Pavord S. et al., 2021). In that study, 97% of cases occurred within 5 to 30 days after vaccination, while 3% presented between 30 and 48 days, typically with isolated venous thromboembolism (such as deep vein thrombosis or pulmonary embolism). The age range at presentation was 18 to 79 years, with a median age of 48 years (interquartile range: 38–56). 56% of patients were under 50 years old, and 85% were under 60. Women were slightly more affected (54% female). The median time from vaccination to symptom onset was 14 days (interquartile range: 10–16). Details regarding patient age, days since vaccination, baseline platelet count, fibrinogen levels, and D-dimer concentrations are provided in Figure 7.

Variable	Value
Age — yr	
Median (IQR)	48 (38–56)
Range	18–79
Sex — no./total no. (%)	
Female	119/217 (55)
Male	98/217 (45)
Race — no./total no. (%)	
White	90/99 (91)
Asian	9/99 (9)
Days since vaccination with ChAdOx1 nCoV-19	
Median (IQR)	14 (10–16)
Range	5–48
Platelet count — per mm ³	
Median (IQR)	47,000 (28,000–76,000)
Range	6,000–344,000
Prothrombin time — sec	
Median (IQR)	13 (10–14)
Range	10–30
Activated partial-thromboplastin time — sec	
Median (IQR)	29 (22–30)
Range	20–57
Fibrinogen level — g/liter	
Median (IQR)	2.2 (1.2–3.1)
Range	0.3–4.4
D-dimer level — FEU	
Median (IQR)	24,000 (8,000–37,000)
Range	5,000–80,000

Figure 7: Data on patient age, days since vaccination and platelet count at baseline and fibrinogen and D-dimer levels are shown in figure

A higher incidence rate of VITT (Vaccine-induced Immune Thrombotic Thrombocytopenia) was reported following the first dose of Vaxzevria (AstraZeneca) in individuals aged 18 to 49 years, compared to the older population (50+ years): 18.0 vs. 10.2 cases per million doses, respectively. There is also evidence suggesting that the incidence rate is slightly higher in females than in males, although this trend was not observed consistently across all age groups, and the differences remain modest. Some authors have argued that the higher incidence in women may reflect a selection bias, as the initial AstraZeneca vaccine recipients were primarily healthcare workers, a profession with a higher proportion of women. As such, the apparent sex difference may not reflect a true biological disparity and is estimated to be around 54% female incidence (Pavord S. et al., 2021).

When assessing risk-benefit ratios, it's important to note that COVID-19 itself appears to significantly increase the risk of atypical venous thromboembolism. In fact, a first-diagnosis incidence of cerebral venous thrombosis (CVT) of 35.3 per million (95% CI: 22.6–55.2) has been observed in COVID-19 patients - significantly higher than in control cohorts with influenza (RR

= 3.83, 95% CI: 1.56–9.41, $p < 0.001$) or among those who received mRNA vaccines (RR = 6.67, 95% CI: 1.98–22.43, $p < 0.001$) (Greinacher A. et al., 2021). In the same study, the incidence of portal vein thrombosis (PVT) was 175.0 per million (95% CI: 143.0–214.1) in patients with COVID-19, again significantly higher than that observed in influenza patients (RR = 1.39, 95% CI: 1.06–1.83, $p = 0.02$) and mRNA vaccine recipients (RR = 7.40, 95% CI: 4.87–11.24, $p < 0.001$) (Torjesen I., 2021).

Within this context, the Pharmacovigilance Risk Assessment Committee (PRAC) of the European Medicines Agency (EMA) deemed a causal link between vaccination and these adverse events as "plausible" (EMA, COVID-19 Vaccine Safety Update: Vaxzevria). This cautious assessment is well-founded, as VITT meets only a subset of the Bradford Hill criteria - a set of nine widely accepted principles used to assess epidemiological evidence of causality between a presumed exposure and a health outcome. Established in 1965 by British epidemiologist Sir Austin Bradford Hill, these criteria are:

- Strength – Stronger associations are more likely to indicate causality;
- Consistency – Reproducibility of results across studies and populations strengthens evidence;
- Specificity – Causality is more likely if a specific cause leads to a specific effect;
- Temporality – The cause must precede the effect;
- Biological gradient (dose-response) – Increased exposure should correlate with increased effect;
- Plausibility – A biologically credible mechanism should link cause and effect;
- Coherence – Consistency between epidemiological and laboratory findings supports the conclusion;
- Experiment – Experimental data, when available, strengthens causality;
- Analogy – Similar exposures producing similar effects can support inference.

In the case of VITT, plausibility is perhaps the most compelling criterion, as it points to a mechanistic paradox involving the simultaneous occurrence of thrombosis and thrombocytopenia - a phenomenon not yet fully understood (Marcucci R. & Marietta M., 2021). In a report published in April 2021, the Centers for Disease Control and Prevention (CDC) in the United States noted that out of 7.98 million doses of the Janssen (Johnson & Johnson) vaccine administered, 15 confirmed cases of TTS (*Thrombosis with Thrombocytopenia Syndrome*) were reported. Notably,

all cases occurred in women, and the majority affected individuals aged 18 to 49.

	Females			Males		
Age group	TTS cases	Doses admin	Reporting rate [‡]	TTS cases	Doses admin	Reporting rate [‡]
18-49 years old	13	1,866,294	7.0 per million	0	1,977,330	0 per million
50+ years old	2	2,125,239	0.9 per million	0	2,010,144	0 per million

Figure 8: Cases of TTS following vaccination with Janssen [Source: CDC, Thrombosis with thrombocytopenia syndrome (TTS) following Janssen COVID-19 vaccine].

Of the 3.99 million doses administered to women, the incidence of VITT was as follows in the various age groups:

	Females		
Age group	TTS cases	Doses admin	Reporting rate [‡]
18-29 years old	3	579,709	5.2 per million
30-39 years old	7	594,215	11.8 per million
40-49 years old	3	692,370	4.3 per million
50-64 years old	2	1,367,529	1.5 per million
65+ years old	0	757,710	0 per million

Figure 9: Reported cases of TTS in women by age group [Source: CDC, Thrombosis with thrombocytopenia syndrome (TTS) following Janssen COVID-19 vaccine].

In the same report, the CDC did not report any cases of cerebral venous sinus thrombosis (CVST) among thrombotic events. However, through the Vaccine Safety Datalink (VSD), 22 cases of venous thromboembolism (VTE) or pulmonary embolism (PE) were reported, of which 16 were subsequently confirmed. Among these cases:

4 had symptom onset prior to vaccination (3 PE, 1 VTE), including one patient with a documented history of thrombocytopenia;

- 1 case had an indeterminate symptom onset;
- 11 cases occurred after vaccination, involving 6 females (2 PE, 4 VTE) and 5 males (1 PE, 4 VTE), with ages ranging between 50 and 79 years. None of these patients had evidence of prior COVID-19 infection, and none presented with thrombocytopenia alongside the thrombotic events (Centers for Disease Control and Prevention, Thrombosis with Thrombocytopenia Syndrome (TTS) following Janssen COVID-19 vaccine).

The German research group that coined the term VITT described a cohort of 11 patients in their April 2021 study published in the New England Journal of Medicine (Greinacher A. et al., 2021). These patients developed one or more thrombotic complications following vaccination with Vaxzevria (ChAdOx1 nCoV-19). The thrombotic events included cerebral venous sinus

thrombosis (CVST) in 9 patients, splenic vein thrombosis in 3 patients, pulmonary embolism in 3 patients and other types of thrombosis in 4 patients. Moreover, 5 patients experienced multiple thrombotic events. The mean age in this group was 36 years (range 22-49), and 9 out of 11 were women. All patients presented with concomitant thrombocytopenia, with an average platelet count around 20,000/mm³ (range 9,000 to 107,000). One patient had a history of von Willebrand disease, tested positive for anticardiolipin antibodies, and was a carrier of the Factor V Leiden mutation (Greinacher A. et al., 2021).

Clinical Presentation of VITT

One of the first studies aiming to characterize the clinical features of VITT was published in April 2021 (Greinacher A. et al., 2021). This study, which included the earliest confirmed or probable VITT cases reported in the UK, defined VITT based on the presence of five diagnostic criteria:

- Symptom onset between 5- and 30-days following SARS-CoV-2 vaccination;
- Evidence of thrombosis;
- Presence of thrombocytopenia (platelet count <150,000/mm³);
- Elevated D-dimer levels (>4000 fibrinogen equivalent units, FEU);
- Positive anti-PF4 antibodies, detected by enzyme-linked immunosorbent assay (ELISA).

Individuals meeting all five criteria were classified as having definite VITT. Cases that did not meet all five criteria were categorized as probable, possible, or unlikely VITT, based on additional clinical judgment. The absence of a single criterion did not exclude the diagnosis entirely, as it could reflect either true absence or a false negative, especially for PF4 antibodies if testing was performed before VITT was widely recognized. Importantly, none of the patients included in the study had been exposed to heparin within the 3 months prior to symptom onset (Greinacher A. et al., 2021).

In the same UK study, VITT patients were typically hospitalized 10 to 12 days post-vaccination with either Vaxzevria (AstraZeneca) or Janssen (Johnson & Johnson). These individuals consistently presented with atypical thrombosis and thrombocytopenia. The most commonly reported events were cerebral venous sinus thrombosis (CVST) and portal vein thrombosis, though more typical thrombotic events such as deep vein thrombosis (DVT) and pulmonary embolism (PE) were also observed. In about 10% of cases, arterial events were reported, including bilateral lower limb ischemia, myocardial infarction, or stroke. Unlike findings from German and Norwegian cohorts, this study did not observe a female predominance. The authors proposed that this difference may be due to the more balanced vaccine distribution between sexes in the UK. They suggest that the higher incidence in females reported elsewhere may be attributable to early

vaccine rollout strategies that prioritized healthcare workers - a workforce with a higher proportion of women (Makris M. et al., 2021).

The coexistence of thrombosis and thrombocytopenia is rare in medicine and occurs in a limited number of conditions, including:

- Thrombotic Thrombocytopenic Purpura (TTP) – a rapidly progressive syndrome characterized by thrombocytopenia and microangiopathic hemolytic anemia. Neurological changes and renal impairment are common. Diagnosis involves laboratory features such as negative direct antiglobulin (Coombs) test and severely reduced ADAMTS13 activity (James N.G.).
- Heparin-Induced Thrombocytopenia (HIT), including its autoimmune variant (aHIT);
- Catastrophic Antiphospholipid Syndrome (CAPS);
- Disseminated Intravascular Coagulation (DIC).

VITT shares several clinical and mechanistic similarities with autoimmune HIT (aHIT). The temporal window of symptom onset, simultaneous occurrence of thrombosis and thrombocytopenia, and the involvement of both venous and arterial sites are features common to both VITT and HIT. Notably, a significant subset of VITT cases exhibited concurrent DIC, characterized by extremely low platelet counts (often $<20,000/\text{mm}^3$), further aligning VITT with the autoimmune form of HIT rather than the classical variant (Marcucci R. & Marietta M., 2021).

Hypotheses on Alternative Pathogenetic Mechanisms Underlying VITT

Serum from patients with VITT typically shows strong reactivity in the PF4-heparin ELISA assay. Interestingly, it has also been observed that in the presence of low doses of low-molecular-weight heparin (LMWH), platelet activation decreases rather than increases, a surprising finding (Greinacher A. et al., 2021). Furthermore, in the same study, in 2 of 11 VITT cases, affinity-purified antibodies against immobilized PF4 or PF4/heparin complexes strongly activated platelets in the presence of PF4 - a feature characteristic of HIT (heparin-induced thrombocytopenia) and used in various diagnostic assays.

A key unresolved question remains: Are these antibodies true autoantibodies targeting endogenous PF4 triggered by the strong inflammatory response to adenoviral vector vaccines? Or are they cross-reactive antibodies induced by the vaccine that incidentally bind to PF4 and activate platelets?

It is well established that adenoviruses, like those used in the Vaxzevria and Janssen vaccines, could bind to platelets and activate them. However, the quantity of adenoviral particles in a 500 μL vaccine dose, administered one to two weeks before the onset of VITT, may not fully account

for the level of platelet activation observed. Nevertheless, interactions between the vaccine and platelets or PF4 could play a crucial role in VITT pathogenesis. One possible trigger for anti-PF4 antibody production is the free DNA contained in the vaccine. In a previous study of Greinacher A. in 2017 on HIT, DNA and RNA extracted from PF4 complexes in HIT patients were shown to induce anti-PF4/heparin antibodies in mice, suggesting a potential immunostimulatory role of nucleic acids.

More recently, a preliminary study has proposed an alternative mechanism. SARS-CoV-2, the virus responsible for COVID-19, is an RNA virus that replicates exclusively in the cytoplasm, bypassing nuclear gene-splicing processes. In contrast, adenoviral vector vaccines deliver DNA to the host cell nucleus, where it is transcribed by the cell's own machinery. Since the viral gene insert (coding for the spike protein) is not optimized for nuclear transcription, it may be subject to aberrant splicing, potentially generating truncated spike protein variants, including soluble forms lacking the C-terminal transmembrane anchor (Kowarz E. et al., 2021). These soluble spike variants can strongly activate endothelial cells expressing ACE2 (Lei Y. et al., 2021). Once the host begins producing anti-spike antibodies, endothelial cells bound to spike proteins may become opsonized, triggering inflammatory damage via antibody-dependent cellular cytotoxicity (ADCC) or complement activation, potentially contributing to the onset of VITT. This hypothesis also aligns with epidemiological data showing a higher prevalence of VITT in young women, who tend to have stronger immune responses than older adults or men. To explain the rarity of VITT, the authors propose that only individuals with certain MHC (*HLA*) genotypes fail to generate sufficient neutralizing antibodies that would otherwise block soluble spike from binding to *ACE2*, thereby preventing downstream inflammation. This mechanism was partially validated through *in-silico* analyses, which identified putative splice donor sites in the spike open reading frame of both the AstraZeneca and Johnson & Johnson vaccines, and further confirmed by *in vitro* studies in HeLa cells showing shorter-than-expected spike protein transcripts. This also explains why VITT has not been observed with mRNA vaccines, which deliver mRNA directly into the cytoplasm, bypassing nuclear splicing. Additionally, it may clarify why VITT appears less frequently with the Johnson & Johnson vaccine compared to AstraZeneca's, as the former contains fewer splice donor sequences (Kowarz E. et al., 2021; Gresele P. et al., 2021).

Additional theories on VITT pathogenesis include:

- Genetically enhanced expression of FcγRIIa receptors on platelets in susceptible individuals, leading to hyper-responsiveness (Gresele P. et al., 2021);
- Altered glycosylation of IgG antibodies induced by vaccination, increasing their affinity for platelet FcγRIIa receptors in some individuals (Kadkhoda K., 2021);

- Circulating adenoviral vectors or pre-existing cross-reactive antibodies to seasonal coronaviruses that form immune complexes, subsequently activating platelets (Chakraborty S. et al., 2021).
- One hypothesis that has largely been dismissed is that prior asymptomatic SARS-CoV-2 infection, leading to circulating anti-spike IgG antibodies capable of activating platelets via FcγRIIa, is a prerequisite for VITT. This is inconsistent with data showing that most VITT patients tested negative for current or past SARS-CoV-2 infection (Douxflis J. et al., 2021).

Other speculative mechanisms include:

- Excessive spike protein expression post-vaccination, potentially activating platelets via ACE2 binding (Shen S. et al., 2021);
- Spike expression by megakaryocytes or platelets, triggering a thrombo-inflammatory storm (Kadkhoda K., 2021; Millington-Burgess S.L. & Harper T.M., 2021).

An additional theory arises from the observation that both ChAdOx1 and Ad26.CoV2.S vaccines contain polysorbate 80 as an excipient (Favaloro E.J., 2021). Polysorbate is a nonionic surfactant that can cross the blood–brain barrier and enhance endothelial uptake of microparticles. Thus, leakage of vaccine vector or excipients like polysorbate into circulation may target cerebral vasculature, promoting local endothelial activation (Choi PHI, 2021). However, given that VITT generally manifests at least a week after vaccination, it is unlikely that either adenoviral particles or excipients remain in circulation at that time. This supports immunologic mechanisms - rather than direct toxicity - as the more plausible explanation (Gresele P. et al., 2021). Ultimately, the precise sequence of pathogenic events leading to VITT - and why it affects only a small subset of individuals with no obvious predisposing factors - remains unclear.

Treatment Options	Comments
High-dose intravenous immunoglobulin (IVIG) (1 g/kg for 2 days)	Increases platelet count and reduces hypercoagulability (based on analogy with autoimmune HIT); inhibits platelet activation by blocking anti-PF4/heparin antibodies.
Anticoagulants commonly used in HIT	Direct oral factor Xa inhibitors (e.g., apixaban, rivaroxaban); Direct thrombin inhibitors (e.g., argatroban, bivalirudin); Indirect factor Xa inhibitors (e.g., danaparoid, fondaparinux).
Platelet transfusion	Should be avoided unless active bleeding is present (theoretically increases risk of thrombosis).
Vitamin K antagonists	Contraindicated during acute thrombocytopenia and disseminated intravascular coagulation (DIC) due to the risk of microthrombosis and protein C depletion.

Table 1: Possible treatments for thrombotic thrombocytopenia induced by ChAdOx1 nCoV19 and Ad26.Cov2.S vaccine

Anti-PF4 Antibodies

Antibodies directed against platelet factor 4 (anti-PF4 antibodies) are currently recognized as the main cause of prothrombotic disorders such as heparin-induced thrombocytopenia (HIT) and vaccine-induced immune thrombotic thrombocytopenia (VITT) following SARS-CoV-2 vaccination. Additionally, it is now established that these antibodies can also cause prothrombotic disorders independent of heparin treatment or adenoviral-based vaccination (Greinacher A., Warkentin T.E., 2023). In the same 2023 study, anti-PF4 antibodies were classified into three distinct types:

- Type 1 anti-PF4 antibodies: These antibodies do not activate platelets and are considered non-pathogenic;
- Type 2 anti-PF4 antibodies: These are pathogenic, platelet-activating antibodies that cause classical HIT. Their prothrombotic activity requires the presence of both PF4 and pharmacological concentrations of heparin;
- Type 3 anti-PF4 antibodies: Like Type 2, these are also platelet-activating and pathogenic. However, they are capable of binding to PF4 independently, without the need for heparin. These antibodies have been identified in patients with VITT and are responsible for triggering thromboinflammation and also in patients with aHIT or in patient after a viral infection.

Both Type 2 and Type 3 antibodies have been associated with the most severe and atypical forms of thrombotic disorders, such as autoimmune HIT (aHIT) and VITT.

Mechanism of Type 2 antibody formation: These antibodies typically develop following heparin exposure. Positively charged PF4 binds to negatively charged heparin, forming complexes that are then targeted by IgM antibodies. Complement factor C3 binds to these immune complexes, facilitating their recognition by B cells through the complement receptor CR2 (CD21). B cells that express receptors for HIT antigens on PF4 then bind to these PF4/heparin complexes, triggering activation and subsequent production of anti-PF4 antibodies (Greinacher A., Warkentin T.E., 2023).

Mechanism of Type 3 antibody formation: Type 3 antibodies are predominantly found in VITT. After vaccination, PF4 interacts with vaccine components and activates B cells. It has been proposed that microvascular injury at the injection site allows vaccine constituents to enter the bloodstream. Adenoviral particles may bind platelets and PF4 (released from activated platelets or endothelium), leading to the same cascade described above: immune complex formation, IgM

and complement binding, and B cell activation. These B cells then produce antibodies specifically targeting PF4-containing complexes.

However, there is a key difference between Type 2 and Type 3 antibodies. In VITT, the binding site on PF4 for Type 3 antibodies is well-characterized - it overlaps with the heparin-binding region but is distinct from the epitope recognized by anti-HIT antibodies. The exact vaccine components that trigger the anti-PF4 immune response and the reason for the loss of immune tolerance post-vaccination remain unknown (Huynh A. et al., Nature, 2021).

Despite the many similarities between these antibody types, an important difference lies in their clonality. Anti-PF4 antibodies in HIT are polyclonal, whereas those in VITT are monoclonal or oligoclonal, with a consistent restriction to a single light-chain immunoglobulin variable region haplotype (Wang JJ et al., Blood, 2022). This suggests a possible genetic predisposition to VITT - unlike HIT, where no genetic susceptibility has been identified (Greinacher A., Warkentin T.E., 2023).

Genetics and Predisposition to VITT

VITT, as already said, is a rare but severe thrombotic complication associated with adenoviral vector vaccines, such as ChAdOx1 nCoV-19 (AstraZeneca) and Ad26.COV2.S (Johnson & Johnson). While most cases occur sporadically, some studies have suggested a potential genetic predisposition, although the current evidence remains preliminary. Recent studies have investigated genetic variants that may be associated with VITT.

One case report described two first cousins with a family history of idiopathic autoimmune thrombocytopenia (ITP), both of whom developed VITT after receiving the Ad26.COV2.S vaccine (Arnold D. M., et al. 2025). Genetic analysis was performed using Next Generation Sequencing (NGS) with a virtual multigene panel based on whole-exome data. The panel included 41 genes associated with genetically determined high risk for thrombotic disorders, including: *ABCG5*, *ABCG8*, *ADAMTS13*, *ANKRD26*, *CYCS*, *DIAPH1*, *ETV6*, *FCGR2A*, *FLII*, *FLNA*, *FYB1*, *GALE*, *GATA1*, *GFIIB*, *GNE*, *GP1BA*, *GP1BB*, *GP9*, *GPR65*, *HOXA11*, *ITGA2B*, *ITGB3*, *MASTL*, *MECOM*, *MPL*, *MYH9*, *NBEAL2*, *ORAI1*, *PRKACG*, *PTPRJ*, *RBM8A*, *RUNX1*, *SLFN14*, *SRC*, *STIM1*, *THPO*, *TPM4*, *TRPM7*, *TUBB1*, *VWF*, and *WAS*.

No pathogenic or likely pathogenic variants were identified, but a homozygous variant in the *FCGR2A* gene (*rs1801274 H131R*) was found. This variant is associated with increased affinity for IgG antibodies and may influence immune responses and susceptibility to VITT.

Another foundational study for genetic research into VITT was conducted in Italy and included 16 patients who had received adenoviral-based vaccines - 14 with ChAdOx1 and 2 with Ad26.COV2.S (Petito E., et al. 2025). Of these, 11 patients (68.75%) were classified as confirmed VITT, while 5 (31.25%) were considered probable VITT. This study combined whole-exome sequencing (WES) with HLA typing, given the gene's polymorphism and known role in autoimmune conditions such as thrombotic thrombocytopenic purpura (TTP), heparin-induced thrombocytopenia (HIT), and immune thrombocytopenia. WES did not reveal a significant difference in the number of genetic variants between the VITT group and healthy controls. However, HLA typing identified three class II HLA alleles - *DPB1*17:01*, *DQA1*05:01*, and *DRB1*11:04* - that were more frequent in VITT patients than in controls. These alleles had not been previously associated with HIT or autoimmune thrombocytopenia, suggesting a distinct HLA distribution pattern in VITT compared to other immune-mediated thrombotic conditions. Moreover, *in-silico* peptide-binding assays identified a peptide derived from PF4 (ITSLEVIKA) as a strong binder of *HLA-DRB1*11:04*. This peptide includes two amino acids (Glu28 and Ala32) previously found within the binding site of VITT-associated anti-PF4 antibodies. These findings support the hypothesis that certain individuals may be genetically predisposed to develop anti-PF4 antibodies in response to adenoviral vector vaccines.

Other studies have explored variants in genes related to coagulation and immune response, such as *MTHFR*, *F11* and *IFNAR2*, but findings have been inconclusive or involved variants of uncertain clinical significance (Mendes-de-Almeida D.P. et al., 2025)

Although some research suggests a potential genetic predisposition to VITT, current evidence is insufficient to identify reliable genetic markers for risk assessment. VITT remains a rare condition, and most cases occur in individuals without known genetic risk factors. Large-scale studies are needed to better understand the genetic mechanisms involved in VITT.

AIM OF THE PROJECT

Despite significant efforts to elucidate the pathophysiological mechanisms underlying vaccine-induced immune thrombotic thrombocytopenia (VITT) - with the PF4-autoantibody complex and its role in activating the FcγRIIA receptor being the most clearly recognized - many aspects of the sequence of events leading to its development remain poorly understood. The identification of specific genetic determinants that predispose individuals to this extremely rare condition could be a key step in uncovering the molecular mechanisms involved in its pathogenesis. The aim of this project is therefore to leverage the potential of Next Generation Sequencing (NGS) technologies - specifically through a Whole Exome Sequencing (WES) approach - to identify potential genetic susceptibility profiles linked to molecular pathways involved in the development of thrombotic and/or thrombocytopenic phenotypes in six individuals diagnosed with VITT.

Given the rarity of this condition, the analysis was focused on rare genetic variants, defined as those with a minor allele frequency (MAF) below 1%. The use of WES in this cohort of six VITT patients - considering both the low prevalence and the distinct clinical features of the disorder - may contribute to expanding our understanding of the sequence of molecular and immunological events that give rise to this clinically significant complication, which emerged in the context of the SARS-CoV-2 pandemic.

METHODS AND MATERIALS

Manual DNA Extraction

The blood sample is collected in a Vacutainer® tube containing the anticoagulant EDTA or citrate. The tubes must be stored at -20 °C until extraction. Of course, the utmost care and precision must be taken in the individual process steps, because the quality of the sample is crucial for the reproducibility and reliability of the later analysis results.

Qiagen FlexiGene® DNA Kit

The FlexiGene® Kit ensures high DNA yield and purity, free of contaminants and inhibitors for subsequent PCR reactions and sequencing. Additionally, it provides a quick and easy method for DNA purification from whole blood. The procedure begins with 0.5 mL of starting material and is performed in a single Eppendorf tube, minimizing waste and the risk of sample mix-ups.

After extraction, the product can be stored at 2-8°C (for short-term storage) or at -20°C (for longer periods). The lysis buffer is added to the sample, causing the cell nuclei and mitochondria to form pellets by centrifugation. The pellet is then resuspended and incubated in a denaturation buffer containing a chaotropic salt and QIAGEN protease. This step helps remove impurities such as proteins.

The DNA is precipitated by adding isopropanol, recovered by centrifugation and washed with 70% ethanol. Finally, the DNA is dried and resuspended in a hydration buffer (10 nM Tris-Cl, pH 8.5).

Protocol:

Pipette 1.25 ml of Buffer FG1 into a 2 ml Eppendorf tube and add 0.5 ml of whole blood; invert the vial at least 10 times.

Centrifuge for 1 min at 12,000 × g.

Discard the supernatant and leave the tube inverted on a clean piece of absorbent paper for 2 min, ensuring that the pellet remains adhered to the bottom of the tube.

In a 1.5 ml Eppendorf tube, prepare a mix consisting of the Protease enzyme (necessary for breaking peptide bonds in proteins) and its reaction buffer, FG2. Dispense 250 µl of the FG2/Protease mix into the vial, close it, and vortex vigorously until the pellet is homogenized and detached from the bottom.

Invert the tube 3 times, place it in a water bath, and incubate at 65°C for 5 min (the sample will change color from red to olive green, indicating successful protein digestion).

Add 250 µl of 100% isopropanol and mix by inversion until the DNA precipitates and becomes visible as a white filamentous clump.

Centrifuge for 3 min at 12,000 × g.

Discard the supernatant and invert the tube onto a clean piece of absorbent paper, ensuring that the pellet does not slip away.

Add 250 µl of 70% ethanol to remove any contaminants and vortex for 5 s.

Centrifuge for 3 min at 12,000 × g.

Discard the supernatant and leave the tube inverted on clean absorbent paper for at least 5 min, ensuring that the pellet remains adhered to the bottom.

Allow the pellet to dry by leaving the tube open to air until all liquid has evaporated (at least 5 min). Be careful not to over-dry the pellet, as this may prevent complete dissolution upon adding the elution buffer (FG3).

Add 200 µl of Buffer FG3, vortex for 5 s, and dissolve the DNA by incubating it in a thermostatic water bath at 65°C for 1 h (if the DNA is not fully dissolved, it is recommended to leave the solution overnight at room temperature).

Qualitative and quantitative evaluation using nanodrop

The sample taken must have good concentration and quality. The fastest and sufficiently reliable way to check this is to use the *UV-Vis NanoDrop™ One/One c spectrophotometer*, which analyzes DNA using the ND1000 v.3.3.0 software. The concentration is determined according to the Lambert-Beer law:

$$C = \frac{OD_{260} \cdot \epsilon}{L}$$

Where:

OD260 is the absorbance at 260 nm, defined as the logarithm of the ratio between I_0 (the intensity of incoming radiation) and I (the intensity of outgoing radiation).

ϵ is the molar extinction coefficient, which depends on the wavelength.

L is the optical path length.

For quality control, the instrument provides two key parameters:

- A260/A280 ratio, which indicates the purity of the nucleic acid. If the value is around 1.8, the DNA is considered pure and free of protein contamination.
- A260/A230 ratio, which detects potential contaminants such as aromatic compounds, phenols, carbohydrates, or peptides. The optimal value is approximately 2.2.

It is important to note that nucleic acids absorb at 260 nm, while proteins absorb at 280 nm. To obtain these values, the spectrophotometer performs multiple scans of the sample within a wavelength range of 220 nm to 350 nm, ensuring it captures the peak absorbance of nucleic acids at 260 nm.



Figure 10: UV-Vis NanoDrop™ One/One c spectrophotometer

Preparation of libraries for NGS analysis

DNA quantification using Qubit fluorometer

After quantifying the genomic DNA (gDNA) samples using the NanoDrop measurement, recording the DNA concentration and the absorbance ratios 260/280 and 260/230, proceed with a second quantification using the **Qubit® Fluorometric Method**.

Ensure the availability of required materials, including: 0.5 ml Qubit® assay tubes, Reagent and buffer from the Qubit® kit, Standards for instrument calibration

Prepare the standards:

Add 190 µL of the working solution to the standard tubes.

Add 10 µL of Qubit® standard to the respective tube.

Vortex for 2-3 seconds, ensuring no bubble formation.

Prepare the sample tubes:

Ensure the total volume (working buffer + sample) is exactly 200 µL.

The sample volume should be between 1–20 µL, with the corresponding working buffer volume

ranging from 180-199 μL .

Vortex for 2-3 seconds.

Incubation and Measurement:

Incubate the tubes at room temperature for 2 minutes.

First, measure the standards, followed by the samples, using the Qubit® fluorometer.



Figure 11: Qubit Fluorimeter

SureSelectXT HS Target Enrichment System

With regard to the genetic susceptibility study in VITT subjects, DNA libraries were constructed using the SureSelectXT HS Target Enrichment System and WES was conducted through NextSeq 500 Illumina Protocol.

Library preparation

In every NGS protocol, preparation of the DNA library, which contains thousands of copies of the selected regions in the panel of interest, is required. This sequencing library was made through Illumina's NextSeq platform, using the SureselectXT HS Target Enrichment System kit and SureSelect XT HS Human All Exon V8 probes from the Agilent company. The SureSelectXT HS target enrichment workflow is summarized in Figure 12.

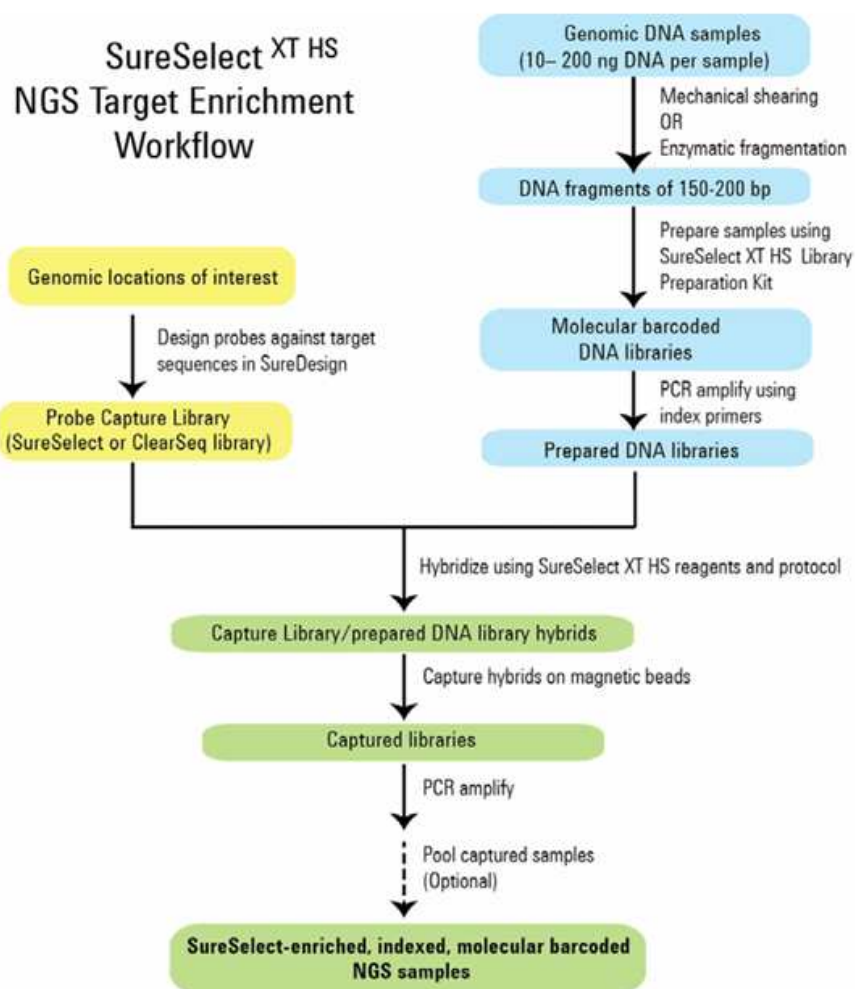


Figure 12: Overall SureSelectXT HS sequencing sample preparation workflow.

STEP 1. ENZYMATIC DNA FRAGMENTATION

In this step, gDNA samples are fragmented using Agilent's SureSelect Enzymatic Fragmentation Kit.

1- In wells of a thermal cycler-compatible strip tube or PCR plate, dilute 10 ng to 200 ng of each gDNA sample with nuclease-free water or 1X Low TE Buffer to a final volume of 7 µl.

2- Thaw the vial of 5X SureSelect Fragmentation Buffer on ice, vortex, then keep on ice.

3- Preprogram a thermal cycler (with the heated lid ON) with the program in Table 2. Immediately pause the program, and keep paused until samples are loaded in step 7.

Step	Temperature	Time
Step 1	37°C	15 minutes for 150 to 200 bp target fragment size
		10 minutes for 180 to 250 bp target fragment size
Step 2	65°C	5 minutes
Step 3	4°C	Hold

Table 2: Thermal cycler program for enzymatic fragmentation.

4- Prepare the appropriate volume of Fragmentation master mix by combining the reagents in Table 3. Mix well by pipetting up and down 20 times or seal the tube and vortex at high speed for 5–10 seconds. Spin briefly to remove any bubbles and keep on ice.

Reagent	Volume for 1 reaction	Volume for 8 reactions (includes excess)	Volume for 24 reactions (includes excess)
5X SureSelect Fragmentation Buffer	2 µl	18 µl	50 µl
SureSelect Fragmentation Enzyme	1 µl	9 µl	25 µl
Total	3 µl	27 µl	75 µl

Table 3: Preparation of Fragmentation master mix.

5- Add 3 µl of the Fragmentation master mix to each sample well containing 7 µl of input DNA.

6- Mix well by pipetting up and down 20 times or cap the wells and vortex at high speed for 5–10 seconds. Spin the samples briefly.

7- Immediately place the plate or strip tube in the thermal cycler and resume the thermal cycling program in Table 2.

8- When the program reaches the 4°C Hold step, remove the samples from the thermal cycler, add 40 µl of nuclease-free water to each sample, and place the samples on ice.

STEP 2. REPAIR AND DA-TAIL THE DNA ENDS

1- Before starting the end-repair protocol, prepare the Ligation master mix to allow equilibration to room temperature before use.

- a) Vortex the thawed vial of Ligation Buffer for 15 seconds at high speed to ensure homogeneity.
- b) Prepare the appropriate volume of Ligation master mix by combining the reagents in Table 4. Slowly pipette the Ligation Buffer into a 1.5-ml Eppendorf tube, ensuring that the full volume is dispensed. Slowly add the T4 DNA Ligase, rinsing the enzyme tip with buffer solution after addition. Mix well by slowly pipetting up and down 15–20 times or seal the tube and vortex at high speed for 10–20 seconds. Spin briefly to collect the liquid.

Keep at room temperature for 30–45 minutes before use on step 3.3.

Reagent	Volume for 1 reaction	Volume for 8 reactions (includes excess)	Volume for 24 reactions (includes excess)
Ligation Buffer (purple cap or bottle)	23 µl	207 µl	598 µl
T4 DNA Ligase (blue cap)	2 µl	18 µl	2 µl
Total	25 µl	225 µl	650 µl

Table 4: Preparation of Ligation master mix.

2- Preprogram a thermal cycler (with the heated lid ON) with the program in Table 5 for the End Repair and dA-Tailing steps. Immediately pause the program, and keep paused until samples are loaded in step 6.

Step	Temperature	Time
Step 1	20°C	15 minutes
Step 2	72°C	15 minutes
Step 3	4°C	Hold

Table 5: Thermal cycler program for End Repair/dA-Tailing

3- Vortex the thawed vial of End Repair-A Tailing Buffer for 15 seconds at high speed to ensure homogeneity. Visually inspect the solution; if any solids are observed, continue vortexing until all solids are dissolved.

4- Prepare the appropriate volume of End Repair/dA-Tailing master mix, by combining the reagents in Table 6. Slowly pipette the End Repair-A Tailing Buffer into a 1.5-ml Eppendorf tube, ensuring that the full volume is dispensed. Slowly add the End Repair-A Tailing Enzyme Mix, rinsing the enzyme tip with buffer solution after addition. Mix well by pipetting up and down 15–20 times or seal the tube and vortex at high speed for 5–10 seconds. Spin briefly to collect the liquid and keep on ice.

Reagent	Volume for 1 reaction	Volume for 8 reactions (includes excess)	Volume for 24 reactions (includes excess)
End Repair-A Tailing Buffer (yellow cap or bottle)	16 µl	144 µl	416 µl
End Repair-A Tailing Enzyme Mix (orange cap)	4 µl	36 µl	104 µl
Total	20 µl	180 µl	520 µl

Table 6: Preparation of End Repair/dA-Tailing master mix.

5- Add 20 µl of the End Repair/dA-Tailing master mix to each sample well containing approximately 50 µl of fragmented DNA. Mix by pipetting up and down 15–20 times using a pipette set to 60 µl or cap the wells and vortex at high speed for 5–10 seconds.

6- Briefly spin the samples, then immediately place the plate or strip tube in the thermal cycler and resume the thermal cycling program in Table 5.

STEP 3. LIGATE THE MOLECULAR-BARCODED ADAPTOR

1- Once the thermal cycler reaches the 4°C Hold step, transfer the samples to ice while setting up this step.

2- Preprogram a thermal cycler (with the heated lid ON) for the Ligation step with the program in Table 7. Immediately pause the program, and keep paused until samples are loaded in step 5.

Step	Temperature	Time
Step 1	20°C	30 minutes
Step 2	4°C	Hold

Table 7: Thermal cycler program for Ligation.

3- To each end-repaired/dA-tailed DNA sample (approximately 70 µl), add 25 µl of the Ligation master mix that was prepared before and kept at room temperature. Mix by pipetting up and down at least 10 times using a pipette set to 85 µl or cap the wells and vortex at high speed for 5–10

seconds. Briefly spin the samples.

4- Add 5 µl of Adaptor Oligo Mix (white capped tube) to each sample. Mix by pipetting up and down 15–20 times using a pipette set to 85 µl or cap the wells and vortex at high speed for 5–10 seconds.

5- Briefly spin the samples, then immediately place the plate or strip tube in the thermal cycler and resume the thermal cycling program in Table 7.

STEP 4. PURIFY THE SAMPLE USING AMPURE XP BEADS

In this step, the DNA libraries are purified using AmpPure XP Beads. Critical purification protocol parameters are summarized for experienced users in Table 8.

Parameter	Value
Volume of RT AMPure XP bead suspension added to each sample well	80 µl
Final elution solvent and volume	35 µl nuclease-free water
Amount of eluted sample transferred to fresh well	Approximately 34.5 µl

Table 8: AMPure XP bead cleanup parameters after adaptor ligation

1- Verify that the AMPure XP beads were held at room temperature for at least 30 minutes before use.

2- Prepare 400 µl of 70% ethanol per sample, plus excess, for use in step 8.

3- Mix the AMPure XP bead suspension well so that the reagent appears homogeneous and consistent in color.

4- Add 80 µl of homogeneous AMPure XP beads to each DNA sample (approximately 100 µl) in the PCR plate or strip tube. Pipette up and down 15–20 times or cap the wells and vortex at high speed for 5–10 seconds to mix.

5- Incubate samples for 5 minutes at room temperature.

6- Put the plate or strip tube into a magnetic separation device. Wait for the solution to clear (approximately 5 to 10 minutes).

7- Keep the plate or strip tube in the magnetic stand. Carefully remove and discard the cleared solution from each well. Do not touch the beads while removing the solution.

8- Continue to keep the plate or strip tube in the magnetic stand while you dispense 200 µl of freshly-prepared 70% ethanol in each sample well.

9- Wait for 1 minute to allow any disturbed beads to settle, then remove the ethanol.

10- Repeat step 8 to step 9 once.

11- Seal the wells with strip caps, then briefly spin the samples to collect the residual ethanol. Return the plate or strip tube to the magnetic stand for 30 seconds. Remove the residual ethanol with a P20 pipette.

12- Dry the samples by placing the unsealed plate or strip tube on the thermal cycler, set to hold samples at 37°C, until the residual ethanol has just evaporated (typically 1–2 minutes).

13- Add 35 µl nuclease-free water to each sample well.

14- Seal the wells with fresh caps, then mix well on a vortex mixer and briefly spin the plate or strip tube to collect the liquid.

15- Incubate for 2 minutes at room temperature.

16- Put the plate or strip tube in the magnetic stand and leave for approximately 5 minutes, until the solution is clear.

17- Remove the cleared supernatant (approximately 34.5 µl) to a fresh PCR plate or strip tube sample well and keep on ice. You can discard the beads at this time.

STEP 5. AMPLIFY THE ADAPTOR-LIGATED LIBRARY

1- Determine the appropriate index assignments for each sample. Use a different index for each sample to be sequenced in the same lane.

2- Preprogram a thermal cycler (with the heated lid ON) with the program in Table 9. Immediately pause the program, and keep paused until samples are loaded in step 6.

Segment	Number of Cycles	Temperature	Time
1	1	98°C	2 minutes
2	8 to 14, based on input DNA quality and quantity (see Table 24)	98°C	30 seconds
		60°C	30 seconds
		72°C	1 minute
3	1	72°C	5 minutes
4	1	4°C	Hold

Table 9: Pre-Capture PCR Thermal Cycler Program

Quality of Input DNA	Quantity of Input DNA	Cycles
Intact DNA from fresh sample	100 to 200 ng	8 cycles
	50 ng	9 cycles
	10 ng	11 cycles
FFPE sample DNA	100 to 200 ng	11 cycles
	50 ng	12 cycles
	10 ng	14 cycles

Table 10: Pre-capture PCR cycle number recommendations

3- Prepare the appropriate volume of pre-capture PCR reaction mix, as described in Table 11, on ice. Mix well on a vortex mixer.

Reagent	Volume for 1 reaction	Volume for 8 reactions (includes excess)	Volume for 24 reactions (includes excess)
5× Herculase II Reaction Buffer	10 µl	90 µl	250 µl
100 mM dNTP Mix	0.5 µl	4.5 µl	12.5 µl
Forward Primer	2 µl	18 µl	50 µl
Herculase II Fusion DNA Polymerase	1 µl	9 µl	25 µl
Total	13.5 µl	121.5 µl	337.5 µl

Table 11: Preparation of Pre-Capture PCR Reaction Mix

4- Add 13.5 µl of the PCR reaction mixture prepared in Table 11 to each purified DNA library sample (34.5 µl) in the PCR plate wells.

5- Add 2 µl of the appropriate SureSelect XT HS Index Primer to each reaction. Cap the wells then vortex at high speed for 5 seconds. Spin the plate or strip tube briefly to collect the liquid release any bubbles.

6- Before adding the samples to the thermal cycler, resume the program in Table 9 to bring the temperature of the thermal block to 98°C. Once the cycler has reached 98°C, immediately place the sample plate or strip tube in the thermal block and close the lid.

STEP 6. PURIFY THE AMPLIFIED LIBRARY WITH AMPURE XP BEADS

In this step, the amplified DNA libraries are purified using AmpPure XP Beads. Critical purification protocol parameters are summarized for experienced users in Table 12.

Parameter	Value
Volume of RT AMPure XP bead suspension added to each sample well	50 µl
Final elution solvent and volume	15 µl nuclease-free water
Amount of eluted sample transferred to fresh well	Approximately 15 µl

Table 12: AMPure XP bead cleanup parameters after pre-capture PCR

- 1- Verify that the AMPure XP beads were held at room temperature for at least 30 minutes before use.
- 2- Prepare 400 µl of 70% ethanol per sample, plus excess, for use in step 8.
- 3- Mix the AMPure XP bead suspension well so that the reagent appears homogeneous and consistent in color.
- 4- Add 50 µl of homogeneous AMPure XP beads to each 50-µl amplification reaction in the PCR plate or strip tube. Pipette up and down 15–20 times or cap the wells and vortex at high speed for 5–10 seconds to mix.
- 5- Incubate samples for 5 minutes at room temperature.
- 6- Put the plate or strip tube into a magnetic separation device. Wait for the solution to clear (approximately 5 minutes).
- 7- Keep the plate or strip tube in the magnetic stand. Carefully remove and discard the cleared solution from each well. Do not touch the beads while removing the solution.
- 8- Continue to keep the plate or strip tube in the magnetic stand while you dispense 200 µl of freshly-prepared 70% ethanol into each sample well.
- 9- Wait for 1 minute to allow any disturbed beads to settle, then remove the ethanol.
- 10- Repeat step 8 and step 9 step once.
- 11- Seal the wells with strip caps, then briefly spin the samples to collect the residual ethanol. Return the plate or strip tube to the magnetic stand for 30 seconds. Remove the residual ethanol with a P20 pipette.
- 12- Dry the samples by placing the unsealed plate or strip tube on the thermal cycler, set to hold samples at 37°C, until the residual ethanol has just evaporated (typically 1–2 minutes).

13- Add 15 μ l nuclease-free water to each sample well.

14- Seal the wells with strip caps, then mix well on a vortex mixer and briefly spin the plate or strip tube to collect the liquid.

15- Incubate for 2 minutes at room temperature.

16- Put the plate or strip tube in the magnetic stand and leave for 2 to 3 minutes, until the solution is clear.

17- Remove the cleared supernatant (approximately 15 μ l) to a fresh PCR plate or strip tube sample well and keep on ice. You can discard the beads at this time.

STEP 7. ASSESS QUALITY AND QUANTITY USING THE 2100 BIOANALYZER AND DNA 1000 ASSAY

The Bioanalyzer 2100 is an instrument equipped with platinum electrodes that enable qualitative and quantitative analysis of nucleic acids in sensitive applications such as NGS. The analysis involves reading samples with High Sensitivity DNA Kit and is based on capillary electrophoresis that takes place inside a particular chip. Each chip contains a set of tightly interconnected microchannels used for separation of nucleic acid fragments based on different molecular weight. At the end of the electrophoretic run, the samples are read according to their fluorescence, and this information is translated into electropherograms.



Figure 13: The Chip Priming Station and the BioAnalyzer 2100.

GEL DYE MIX PREPARATION:

1- Equilibrate High Sensitivity DNA Dye concentrate and High Sensitivity DNA Gel Matrix reagents at room temperature for 30 min;

2- Add 15 μ l of High Sensitivity DNA Dye concentrate to the vial of High Sensitivity DNA Gel Matrix;

- 3- Vortex the solution well and centrifuge briefly; transfer to spin filter;
- 4- Centrifuge at $2240g \pm 20\%$ for 10 min. Protect the solution from light and store it at 4°C .

“CHIP PRIMING STATION” SETTING:

- 1- Set the syringe lock lever to the lowest position;
- 2- Check that the base is in position “C”.

LOADING THE BIOANALYZER CHIP:

- 1- Transfer 9 μl of Gel-Dye Mix into the Chip at the position marked by the black G (Figure 14). You lower the syringe until it is wedged into the block. Start the timer for 60 seconds (time needed for the matrix to spread out on the Chip). Release the syringe block and let it rise spontaneously until it stops, at which point manually with unexaggerated speed you raise the syringe plunger to 1 ml;
- 2- Load 9 μl of Gel-Dye Mix into the other 3 G;
- 3- Load 5 μl of DNA marker into the other wells of the Chip;
- 4- Load 1 μl of DNA ladder into the indicated well;
- 5- Load 1 μl of sample into the remaining wells (row 1: samples 1-3, row 2: samples 3-6, row 3: samples 7-9, row 4: samples 10-11);
- 6- Vortex the chip for 1 minute at 2400rpm;
- 7- Check for bubbles in the Chip before loading it into the Bioanalyzer;
- 8- Select from the computer dsDNA, High Sensitivity DNA assay, the number of samples loaded and their identifier. At this point select “start”
- 9- Verify that the electropherogram shows the peak of DNA fragment size positioned between 300 to 400 bp. Sample electropherograms are shown in Figure 15.

To note that the Bioanalyzer is volume-sensitive so all wells must be loaded. If there are empty wells left because there are less than 11 samples, load an equal volume of markers in their place.

After analysing the Bioanalyzer results, continue with the SureSelect XT from Step 8.



Figure 14: High Sensitivity DNA chip

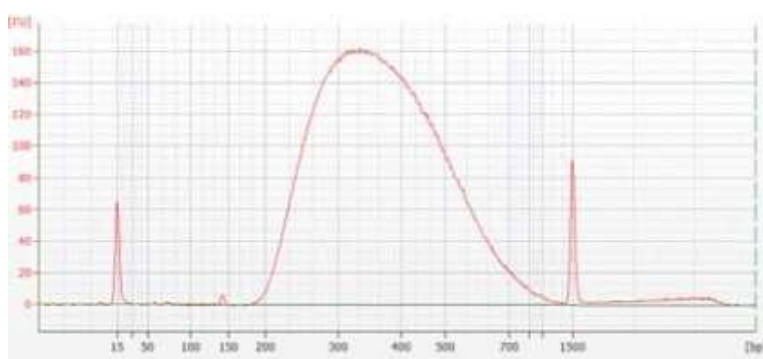


Figure 15: Pre-capture library prepared from gDNA sample analyzed using a DNA 1000 Bioanalyzer assay.

STEP 8. HYBRIDIZE DNA SAMPLES TO THE PROBE

In this step, the prepared gDNA libraries are hybridized to a target- specific probe. For each sample library prepared, you do one hybridization and capture. Do not pool samples at this stage. The hybridization reaction requires 500–1000 ng of prepared DNA in a volume of 12 μ l. Use the maximum amount of prepared DNA available within this range.

1- Preprogram a thermal cycler (with the heated lid ON) with the program in Table 13. Immediately pause the program, and keep paused until samples are loaded.

Segment #	Number of cycles	Temperature	Time
1	1	95°C	5 minutes
2	1	65°C	10 minutes
3	1	65°C	1 minute (Pause cycler here for reagent addition)
4	60	65°C	1 minute
		37°C	3 seconds

5	1	65°C	Hold briefly until ready to begin capture steps
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Table 13: Pre-programmed thermal cycler program for Hybridization

2- Place 500–1000 ng of each prepared gDNA library sample into the hybridization plate or strip tube wells and then bring the final volume in each well to 12 µl using nuclease-free water. Use the maximum possible amount of each prepped DNA, within the 500–1000 ng range.

3- To each DNA library sample well, add 5 µl of SureSelect XT HS and XT Low Input Blocker Mix (blue cap). Seal the wells then vortex at high speed for 5 seconds. Spin the plate or strip tube briefly to collect the liquid and release any bubbles.

4- Transfer the sealed sample plate or strip to the thermal cycler and resume the thermal cycling program (Table 13), allowing the cycler to complete Segments 1 and 2 of the program. Important: The thermal cycler must be paused during Segment 3 to allow additional reagents to be added to the Hybridization wells in step 7. During Segments 1 and 2 of the thermal cycling program, begin preparing the additional hybridization reagents as described in step 5 and step 6 below. If needed, you can finish these preparation steps after pausing the thermal cycler in Segment 3.

5- Prepare a 25% solution of SureSelect RNase Block (1 part RNase Block to 3 parts water) according to Table 14. Prepare the amount required for the number of hybridization reactions in the run, plus excess. Mix well and keep on ice.

Reagent	Volume for 1 reaction	Volume for 8 reactions (includes excess)	Volume for 24 reactions (includes excess)
SureSelect RNase Block	0.5 µl	4.5 µl	12.5 µl
Nuclease-free water	1.5 µl	13.5 µl	37.5 µl
Total	2 µl	18 µl	50 µl

Table 14: Preparation of RNase Block solution

6- Prepare the Probe Hybridization Mix appropriate for your probe design size. Use Table 15 for probes <3 Mb. Combine the listed reagents at room temperature. Mix well by vortexing at high speed for 5 seconds then spin down briefly. Proceed immediately to step 7.

Reagent	Volume for 1 reaction	Volume for 8 reactions (includes excess)	Volume for 24 reactions (includes excess)
25% RNase Block solution (from step 5)	2 µl	18 µl	50 µl

Probe	2 µl	18 µl	50 µl
SureSelect Fast Hybridization Buffer	6 µl	54 µl	150 µl
Nuclease-free water	3 µl	27 µl	75 µl
Total	13 µl	117 µl	325 µl

Table 15: Preparation of Probe Hybridization Mix for probes <3 Mb

7- Once the thermal cycler starts Segment 3 of the program in Table 13 (1 minute at 65°C), pause the program. With the cycler paused, and while keeping the DNA + Blocker samples in the cycler, transfer 13 µl of the room-temperature Probe Hybridization Mix from step 6 to each sample well. Mix well by pipetting up and down slowly 8 to 10 times. The hybridization reaction wells now contain approximately 30 µl.

8- Seal the wells with fresh caps. Make sure that all wells are completely sealed. Vortex briefly, then spin the plate or strip tube briefly to remove any bubbles from the bottom of the wells. Immediately return the plate or strip tube to the thermal cycler.

9- Resume the thermal cycling program to allow hybridization of the prepared DNA samples to the probe.

STEP 9. PREPARE STREPTAVIDIN-COATED MAGNETIC BEADS

1- Vigorously resuspend the Dynabeads MyOne Streptavidin T1 magnetic beads on a vortex mixer. The magnetic beads settle during storage.

2- For each hybridization sample, add 50 µl of the resuspended beads to wells of a fresh PCR plate or a strip tube.

3- Wash the beads:

- Add 200 µl of SureSelect Binding Buffer.
- Mix by pipetting up and down 20 times or cap the wells and vortex at high speed for 5–10 seconds then spin down briefly.
- Put the plate or strip tube into a magnetic separator device.
- Wait at least 5 minutes or until the solution is clear, then remove and discard the supernatant.
- Repeat step a through step d two more times for a total of 3 washes.

4- Resuspend the beads in 200 µl of SureSelect Binding Buffer.

STEP 10. CAPTURE THE HYBRIDIZED DNA USING STREPTAVIDIN-COATED BEADS

1- After all streptavidin bead preparation steps are complete, and with the hybridization thermal

cycling program in the final hold segment (see Table 13), transfer the samples to room temperature.

2- Immediately transfer the entire volume (approximately 30 μ l) of each hybridization mixture to wells containing 200 μ l of washed streptavidin beads using a multichannel pipette. Pipette up and down 5–8 times to mix then seal the wells with fresh caps.

3- Incubate the capture plate or strip tube on a 96-well plate mixer, mixing vigorously (at 1400–1800 rpm), for 30 minutes at room temperature. Make sure the samples are properly mixing in the wells.

4- During the 30-minute incubation for capture, prewarm SureSelect Wash Buffer 2 at 70°C as described below.

a) Place 200- μ l aliquots of Wash Buffer 2 in wells of a fresh 96-well plate or strip tubes. Aliquot 6 wells of buffer for each DNA sample in the run.

b) Cap the wells and then incubate in the thermal cycler, with heated lid ON, held at 70°C until used in step 9.

5- When the 30-minute incubation period initiated in step 3 is complete, spin the samples briefly to collect the liquid.

6- Put the plate or strip tube in a magnetic separator to collect the beads. Wait until the solution is clear, then remove and discard all of the supernatant.

7- Resuspend the beads in 200 μ l of SureSelect Wash Buffer 1. Mix by pipetting up and down 15–20 times, until beads are fully resuspended.

8- Put the plate or strip tube in the magnetic separator. Wait for the solution to clear (approximately 1 minute), then remove and discard all of the supernatant.

9- Remove the plate or strip tubes from the magnetic separator and transfer to a rack at room temperature. Wash the beads with Wash Buffer 2, using the protocol steps below.

a) Resuspend the beads in 200 μ l of 70°C prewarmed Wash Buffer 2. Pipette up and down 15–20 times, until beads are fully resuspended.

b) Seal the wells with fresh caps and then vortex at high speed for 8 seconds. Spin the plate or strip tube briefly to collect the liquid without pelleting the beads. Make sure the beads are in suspension before proceeding.

c) Incubate the samples for 5 minutes at 70°C in the thermal cycler with the heated lid ON.

d) Put the plate or strip tube in the magnetic separator at room temperature.

e) Wait 1 minute for the solution to clear, then remove and discard the supernatant.

f) Repeat step a through step e five more times for a total of 6 washes.

10- After verifying that all wash buffer has been removed, add 25 µl of nuclease-free water to each sample well. Pipette up and down 8 times to resuspend the beads. Keep the samples on ice until they are used.

STEP 11. AMPLIFY THE CAPTURED LIBRARIES

In this step, the SureSelect- enriched DNA libraries are PCR amplified.

Prepare one amplification reaction for each DNA library.

1- Preprogram a thermal cycler (with the heated lid ON) with the program in Table 16. Immediately pause the program, and keep paused until samples are loaded in step 5.

Segment	Number of Cycles	Temperature	Time
1	1	98°C	2 minutes
2	12 (Probes 0.2–3 Mb)	98°C	30 seconds
		60°C	30 seconds
		72°C	1 minute
3	1	72°C	5 minutes
4	1	4°C	Hold

Table 16: Post-capture PCR thermal cycler program

2- Prepare the appropriate volume of PCR reaction mix, as described in Table 17, on ice. Mix well on a vortex mixer.

Reagent	Volume for 1 reaction	Volume for 8 reactions (includes excess)	Volume for 24 reactions (includes excess)
Nuclease-free water	12.5 µl	112.5 µl	312.5 µl
5× Herculanase II Reaction Buffer	10 µl	90 µl	250 µl
Herculanase II Fusion DNA Polymerase	1 µl	9 µl	25 µl
100 mM dNTP Mix	0.5 µl	4.5 µl	12.5 µl
SureSelect Post-Capture	1 µl	9 µl	25 µl

Primer Mix			
Total	25 µl	225 µl	625 µl

Table 17: Preparation of post-capture PCR Reaction mix

3- Add 25 µl of the PCR reaction mix prepared in Table 17 to each sample well containing 25 µl of bead-bound target-enriched DNA.

4- Mix the PCR reactions well by pipetting up and down until the bead suspension is homogeneous. Avoid splashing samples onto well walls; do not spin the samples at this step.

5- Place the plate or strip tube in the thermal cycler and resume the thermal cycling program in Table 16.

6- When the PCR amplification program is complete, spin the plate or strip tube briefly. Remove the streptavidin-coated beads by placing the plate or strip tube on the magnetic stand at room temperature. Wait 2 minutes for the solution to clear, then remove each supernatant (approximately 50 µl) to wells of a fresh plate or strip tube. The beads can be discarded at this time.

STEP 12. PURIFY THE AMPLIFIED CAPTURED LIBRARIES USING AMPURE XP BEADS

In this step, the amplified enriched DNA libraries are purified using AmpPure XP Beads.

1- Let the AMPure XP beads come to room temperature for at least 30 minutes.

2- Prepare 400 µl of fresh 70% ethanol per sample, plus excess, for use in step 8.

3- Mix the AMPure XP bead suspension well so that the suspension appears homogeneous and consistent in colour.

4- Add 50 µl of the homogeneous AMPure XP bead suspension to each amplified DNA sample (approximately 50 µl) in the PCR plate or strip tube. Mix well by pipetting up and down 15–20 times or cap the wells and vortex at high speed for 5–10 seconds. Check that the beads are in a homogeneous suspension in the sample wells. Each well should have a uniform colour with no layers of beads or clear liquid present.

5- Incubate samples for 5 minutes at room temperature.

6- Put the plate or strip tube on the magnetic stand at room temperature. Wait for the solution to clear (approximately 3 to 5 minutes).

7- While keeping the plate or tubes in the magnetic stand, carefully remove and discard the cleared solution from each well. Do not disturb the beads while removing the solution.

8- Continue to keep the plate or tubes in the magnetic stand while you dispense 200 µl of freshly-

prepared 70% ethanol in each sample well.

9- Wait for 1 minute to allow any disturbed beads to settle, then remove the ethanol.

10- Repeat step 8 and step 9 once for a total of two washes. Make sure to remove all of the ethanol at each wash step.

11- Seal the wells with strip caps, then briefly spin to collect the residual ethanol. Return the plate or strip tube to the magnetic stand for 30 seconds. Remove the residual ethanol with a P20 pipette.

12- Dry the samples by placing the unsealed plate or strip tube on the thermal cycler, set to hold samples at 37°C, until the residual ethanol has just evaporated (typically 1–2 minutes).

13- Add 25 µl of nuclease-free water to each sample well.

14- Seal the sample wells, then mix well on a vortex mixer and briefly spin to collect the liquid without pelleting the beads.

15- Incubate for 2 minutes at room temperature.

16- Put the plate or strip tube in the magnetic stand and leave for 2 minutes or until the solution is clear.

17- Remove the cleared supernatant (approximately 25 µl) to a fresh well. You can discard the beads at this time.

STEP 13. ASSESS SEQUENCING LIBRARY DNA QUANTITY AND QUALITY USING THE AGILENT 2100 BIOANALYZER AND HIGH SENSITIVITY DNA ASSAY

Use the Bioanalyzer High Sensitivity DNA Assay to analyze the amplified captured DNA. Perform the assay according to the High Sensitivity DNA Kit Guide.

1- Set up the 2100 Bioanalyzer instrument as instructed in the reagent kit guide.

2- Prepare the chip, samples and ladder as instructed in the reagent kit guide, using 1 µl of each sample for the analysis.

3- Load the prepared chip into the instrument and start the run within five minutes after preparation.

4- Verify that the electropherogram shows the peak of DNA fragment size positioned between 200 and 400 bp. Sample electropherograms are shown in Figure 16.

5- Measure the concentration of each library by integrating under the entire peak. For accurate quantification, make sure that the concentration falls within the linear range of the assay.

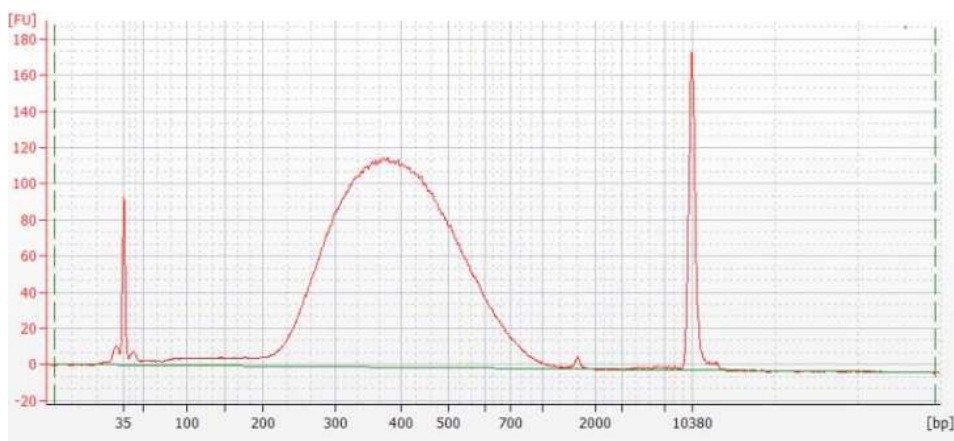


Figure 16: Post-capture library prepared from a high-quality gDNA sample analyzed using a Bioanalyzer system High Sensitivity DNA assay.

STEP 14. POOL SAMPLES FOR MULTIPLEXED SEQUENCING

The number of indexed libraries that may be multiplexed in a single sequencing lane is determined by the output specifications of the platform used, together with the amount of sequencing data required for your research design. Calculate the number of indexes that can be combined per lane, according to the capacity of your platform and the amount of sequencing data required per sample. Combine the libraries such that each index- tagged sample is present in equimolar amounts in the pool using the following method:

Starting with samples at different concentrations, add the appropriate volume of each sample to achieve equimolar concentration in the pool, then adjust the pool to the desired final volume using Low TE. The formula below is provided for determination of the amount of each indexed sample to add to the pool.

$$V_{INDEXED\ SAMPLE} = \frac{V_f \times C_f}{\# \times C_i}$$

V_f = Final desired volume of pool
 C_f = Final desired concentration of pool (4 nM)
 $\#$ = number of indexed samples
 C_i = Initial concentration of each indexed sample

NextSeq 500 Illumina protocol

Dilution and denaturation of libraries for sequencing on NextSeq Illumina Platform

This procedure denatures and dilutes libraries to a final loading volume of 1.3 ml at a recommended concentration of 1.8 pM for high output kits and 1.5 pM for mid output kits. In practice, loading concentration can vary depending on library preparation and quantification methods.

Prepare a Fresh Dilution of NaOH

1- Combine the following volumes in a microcentrifuge tube:

- a) Laboratory-grade water (800 μ l)
- b) Stock 1.0 N NaOH (200 μ l)

The result is 1 ml of 0.2 N NaOH.

2- Invert the tube several times to mix.

NOTE: Use the fresh dilution within 12 hours.

Prepare HT1

1- Remove HT1 from -25°C to -15°C storage and thaw at room temperature.

2- Store at 2°C to 8°C until you are ready to dilute denatured libraries.

Prepare RSB

1- Remove the tube of RSB from -25°C to -15°C storage and thaw at room temperature.

2- Store thawed RSB at 2°C to 8°C until you are ready to dilute libraries.

NOTE: In place of RSB, you can use 10 mM Tris-HCl, pH 8.5 with 0.1% Tween 20

Denature Libraries

1- Combine 5 μ l of library and 5 μ l of freshly diluted 0.2 N NaOH in a microcentrifuge tube.

2- Vortex briefly and then centrifuge at $280 \times g$ for 1 minute.

3- Incubate at room temperature for 5 minutes.

4- Add 5 μ l of 200 mM Tris-HCl, pH 7

5- Vortex briefly and then centrifuge at $280 \times g$ for 1 minute.

Dilute Denatured Libraries to 20 pM

1- Add the 985 μ l of prechilled HT1 to the tube of denatured libraries. The result is a 20 pM

denatured library.

2- Vortex briefly and then centrifuge at $280 \times g$ for 1 minute.

3- Place the 20 pM libraries on ice until you are ready to proceed to final dilution.

Dilute Libraries to Loading Concentration

1- Dilute the denatured 20 pM library solution to 1.5 pM as follows:

a) Denatured library solution (97 μ l)

b) Prechilled HT1 (1203 μ l)

The total volume is 1.3 ml at 1.5 pM.

2- Invert to mix and then pulse centrifuge.

Sequencing process on Illumina NextSeq 500

Prepare the Reagent Cartridge

1- Remove the reagent cartridge from -25°C to -15°C storage.

2- Thaw in a room temperature water bath until thawed (~60 minutes). Do not submerge the cartridge.

3- Gently tap on the bench to dislodge water from the base, and then dry the base.

4- Invert the cartridge five times to mix reagents.

5- Inspect positions 29, 30, 31, and 32 to make sure that reagents are thawed.

6- Gently tap on the bench to reduce air bubbles.

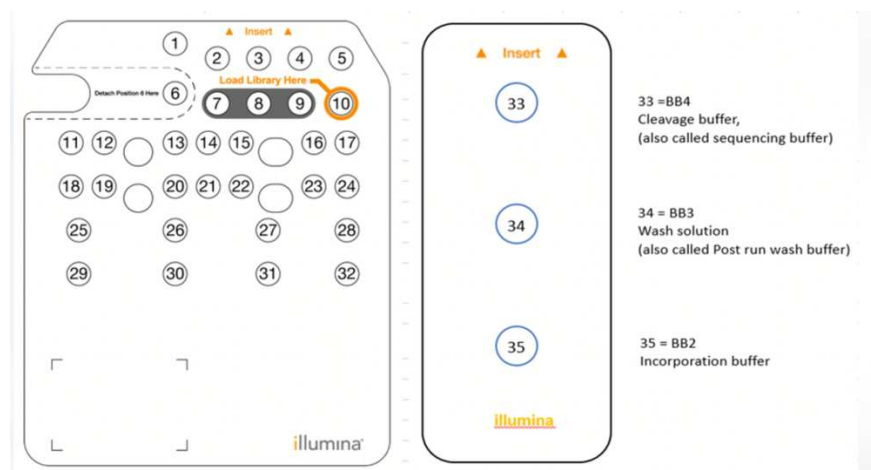


Figure 17: NextSeq 500 reagent cartridge with reserved reservoirs.

Prepare the Flow Cell

- 1- Remove a new flow cell package from 2°C to 8°C storage.
- 2- Remove the flow cell from the foil package
- 3- Set the unwrapped flow cell package aside at room temperature for 30 minutes.
- 4- Open the clear plastic clamshell package and remove the flow cell.
- 5- Clean the glass surface of the flow cell with a lint-free alcohol wipe. Dry the glass with a low-lint lab tissue.

Load Libraries onto the Reagent Cartridge

- 1- Clean the foil seal covering reservoir #10 labeled Load Library Here using a low-lint tissue.
- 2- Pierce the seal with a clean 1 ml pipette tip.
- 3- Load 1.3 ml of prepared 1.8 pM libraries into reservoir #10. Avoid touching the foil seal as you dispense the libraries.

Set Up a Sequencing Run

- 1- From the Home screen, select Experiment.
- 2- On the Select Assay screen, select Sequence.

The Sequence command opens the imaging compartment door, releases consumables from a previous run, and opens the series of run setup screens. A short delay is normal.

When setting up a sequencing run, you select one of the following run modes to determine where to enter run information and how to analyze data.

Run Mode	Run Information	Data Analysis
Local Run Manager	Enter in Local Run Manager	The software saves data to the specified output folder for automatic analysis in Local Run Manager.
Manual	Enter in the NCS (manual run mode)	The software saves data to a specified output folder for later analysis off the instrument.

Table 18: Run modes

Then, you can load the Flow Cell, the Buffer Cartridge and the Reagent Cartridge. After the run, there is an Automatic Post-Run Wash.

Bioinformatic analysis of NextSeq 500 sequencing results

Concerning the Assembly and Variant Calling phases, reads were aligned with the human reference hg19 genome using the Burrows–Wheeler aligner¹⁸⁸. Fast and accurate short read alignment with Burrows–Wheeler transform¹⁶ was performed, and aligned reads were inspected with the IGV software (Broad Institute) (Thorvaldsdottir H. et al., 2013). The variant calling for the identification of nucleotide variants was performed using the Genome Analysis Toolkit HaplotypeCaller Module (McKenna A. et al., 2010). Coverage statistics and the average depth of coverage were computed using custom scripts in the R software environment [<https://www.r-project.org/>].

Rare SNVs and InDels were selected by removing all variants with no functional impact (intronic and missense) and with allelic frequency smaller than 0.0001. For each patient, all genes affected by rare variants were analyzed for overrepresentation analysis by using the “gene list” analysis tool of the Reactome database (reactome.org). Reactome pathways with entities FDR smaller than 0.05 were considered statistically significant.

Genes for variants analysis have been selected according to gene ontology biological processes (blood coagulation [GO:0007596] and fibrinolysis [GO:0042730], platelet activation [GO:0030168] and aggregation [GO:0070527], integrin-mediated signaling pathway [GO:0007229], inflammatory response [GO:0006954], and Human Phenotype Ontology [autoimmune thrombocytopenia: HP:0001973]).

Variant prioritization phase was carried out as follows: nonsynonymous variants with a minor allele frequency (MAF) < 0.01 in publicly available resources (dbSNP; in 1000 Genomes Project or Genome Aggregation Database) were considered. The pathogenic effect of the missense/splicing mutations was estimated using PROVEAN, PolyPhen-2, MutationTaster, FATHMM, NetGene2, ASSP, and SpliceAI tools. Attribution of pathogenicity was performed according to the American College of Medical Genetics (ACMG) guidelines (Richards S. et al., 2015).

RESULTS

The aim of this thesis was to evaluate the whole exome (WES, Whole Exome Sequencing) in patients diagnosed with vaccine-induced immune thrombotic thrombocytopenia (VITT) following SARS-CoV-2 vaccination.

Between February and June 2021, several suspected VITT cases were referred to the Center for Atherothrombotic Diseases, part of the Department of Experimental and Clinical Medicine of the University of Florence and the Careggi University Hospital (Head of Unit: Prof. Rossella Marcucci). Only patients with a possible or definite diagnosis of VITT were enrolled in a prospective registry.

The cohort therefore included 10 patients (7 females and 3 males), with a median age of 63.5 years [IQR 41–78]. The median interval between vaccination and clinical onset was 8 days [IQR 6–23].

All patients with definite VITT presented thrombotic manifestations, seven developed venous thrombosis, typically involving multiple sites, and three of them also exhibited concomitant arterial thrombotic events. Two patients had multiple arterial thrombotic events only. Four patients (40%) also experienced major hemorrhagic complications, all affecting the intracranial region - resulting in death in two cases and severe disability in the others. The last patient (P10) was classified as a case of possible VITT (Lotti E. et al, 2024).

Most patients had no major risk factors for venous thromboembolism (VTE). Only one had a previous VTE episode (a pulmonary embolism following a hip fracture). Thrombophilia risk factors (FV Leiden polymorphism, FII G20210A polymorphism, AT, PS and PC deficiencies, elevated FVIII levels, hyperhomocysteinemia, and antiphospholipid antibodies) were excluded in all patients. The most frequent cardiovascular risk factor was arterial hypertension (Lotti E. et al, 2024).

All patients developed thrombocytopenia with a median platelet count at the nadir of 23,500/mm³ [IQR 17,500-50,000/mm³]. Median D-Dimer level at the presentation was 24,583 ng/ml [IQR 5,141-58,881 ng/mL]. At the time of diagnosis, the median anti-PF4 antibodies quantitative titer resulted extremely high (2,192 ODs) [IQR 1,260-3,084], and it was associated with a positive functional test in all definite VITT (Lotti E. et al, 2024).

Molecular characterization through next-generation sequencing (NGS) was performed on six of the ten patients at the Advanced Genetic-Molecular Laboratory (Scientific Lead: Prof. Betti Giusti) within the Atherothrombotic Diseases Center. All six patients were women of Caucasian descent, with a mean age of 64.2 ± 13.8 years. Among them, five patients (VITT02, VITT18, VITT05, VITT06, VITT21) had received the VAXZEVRIA (AstraZeneca) vaccine, while one patient (VITT03) had received the JANSSEN (Johnson & Johnson) vaccine.

Table 19 presents the clinical data of the six VITT patients included in the study, highlighting

arterial and/or venous thrombotic events, hemorrhagic complications, and significant neurological damage observed during post-hospitalization follow-up.

Patient VITT05 (75 years old) developed thrombocytopenia three days after vaccination and was promptly treated with a combination of immunoglobulins, anticoagulants, plasma, and corticosteroids. Despite progressive normalization of coagulation parameters and platelet count, she experienced severe venous thrombotic events. Her condition deteriorated due to the worsening of a subdural hematoma, cerebral hemorrhages, and cerebral thromboses, ultimately leading to her death.

Patient VITT18, the youngest in the cohort (42 years old), was also promptly treated following suspicion of VITT. A few days after the onset of thrombocytopenia, she developed severe hemorrhagic complications and a hematoma, which required surgical intervention. Subsequently, the appearance of a brain abscess necessitated a second surgery, further complicating her clinical course and requiring rehabilitation therapy.

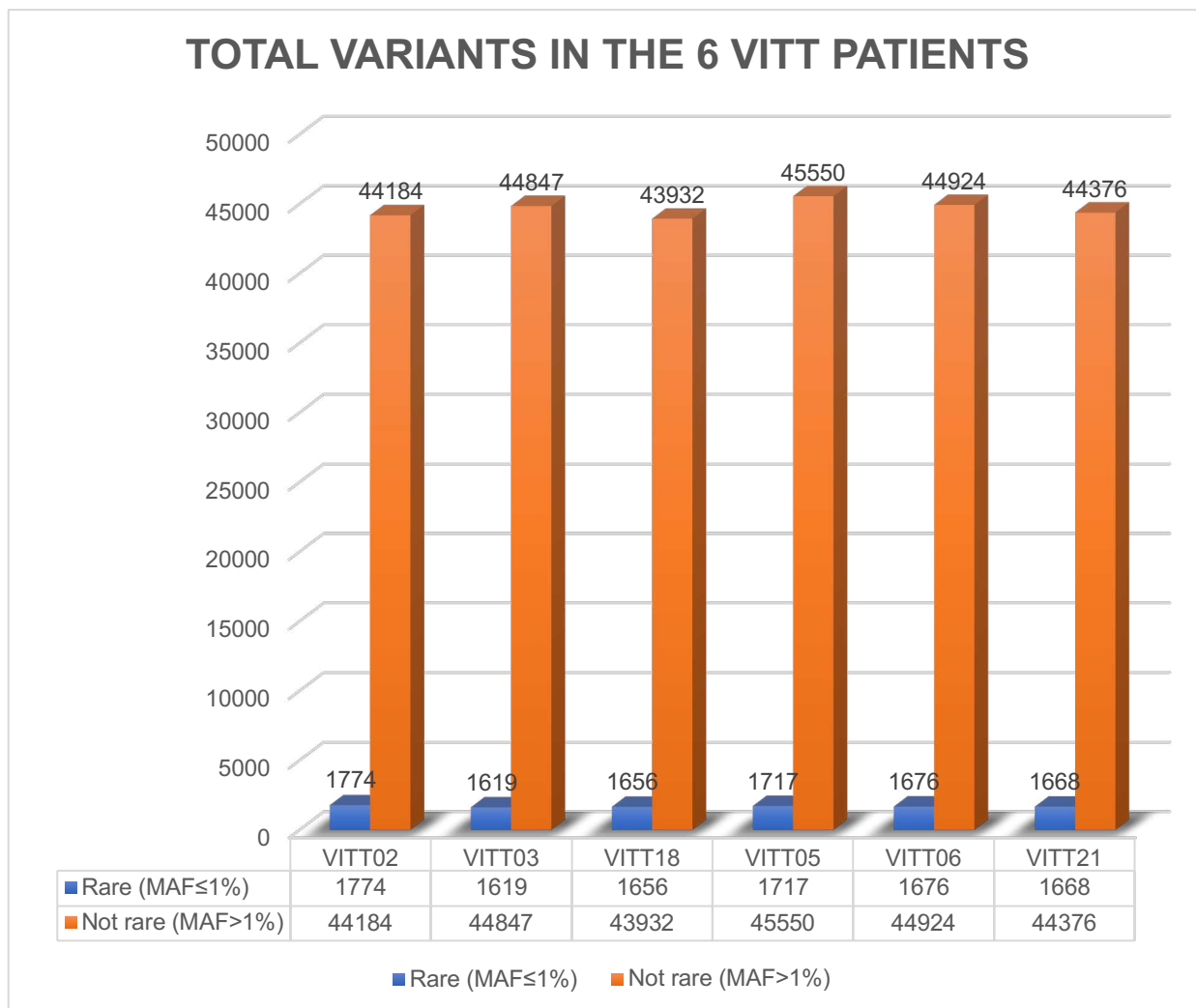
In both patients, despite normalization of platelet counts and coagulation tests, the cerebral hemorrhages - either concurrent with or resulting from the VITT phenotype - contributed significantly to clinical deterioration.

Patients VITT02 and VITT06 experienced both venous and arterial thromboses, which resolved following appropriate therapeutic intervention.

Patients VITT03 and VITT21 had a more favorable clinical course, developing only mild venous thromboses. No apparently significant demographic or clinical differences were observed between patient VITT03, who received the adenoviral JANSSEN vaccine, and the five other patients who received the adenoviral VAXZEVRIA vaccine.

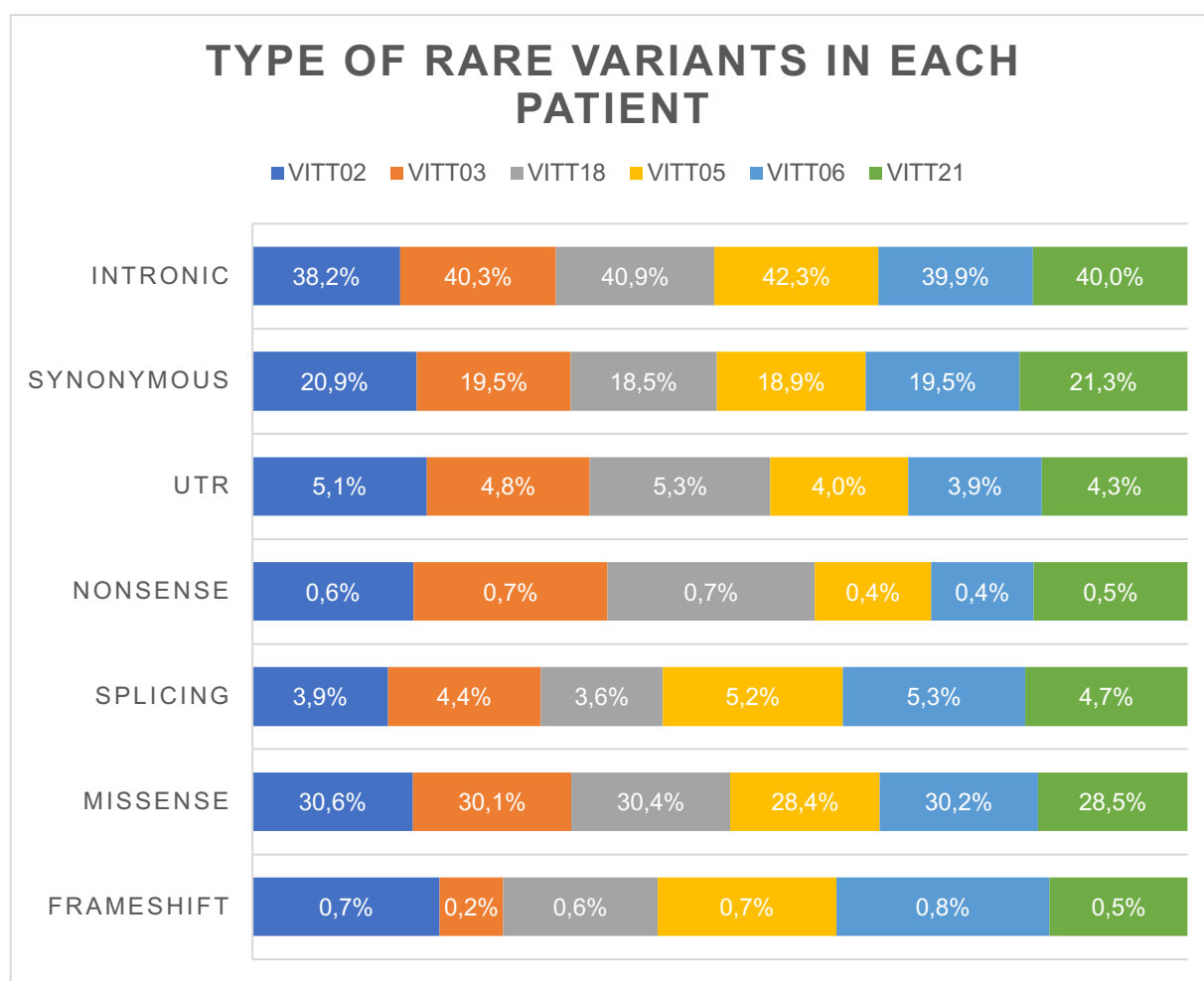
Table 19: Demographic and clinical characteristics of our Study Population (6 patients completely analyzed, also with WES)

PATIENT	AGE (years old)	Remote Pathological History	Venous Thrombotic Manifestations	Arterial Thrombotic Manifestations	Haemorrhagic Complications	Discharge with Disability	Death
VITT02	57 years old	No personal or family history of haemorrhage or thrombotic and/or vascular disorders has been reported	Pulmonary thromboembolism; Portal thrombosis	Abdominal aorta; Splenic artery	NO	NO	NO
VITT03	78 years old	Arterial hypertension, dyslipidaemia, chronic arteriopathy obliterans of the lower limbs on therapy with Aspirin 100 mg/day. No personal or family history of haemorrhage or thrombotic and/or vascular disorders reported	Multiple bilateral venous thrombosis of the lower limbs	/	NO	NO	NO
VITT18	42 years old	No personal or family history of haemorrhage or thrombotic and/or vascular disorders has been reported	/	Middle and anterior cerebral artery, ophthalmic artery, retinal artery (right)	Haemorrhage in the entire right cerebral hemisphere with uncal herniation	YES	NO
VITT05	75 years old	Arterial hypertension, osteoporosis, polymyalgia rheumatica. No personal or family history of haemorrhage or thrombotic and/or vascular disorders has been reported	Portal thrombosis; Cerebral venous sinus thrombosis	/	Portal thrombosis; Cerebral venous sinus thrombosis	YES	YES
VITT06	73 years old	Dyslipidaemia, hypertension, previous appendectomy and cholecystectomy. No personal or family history of haemorrhage or thrombotic and/or vascular disorders has been reported.	Cerebral venous sinus thrombosis; Left pulmonary thromboembolism; Left renal vein	Extensive cerebral ischaemic strokes; Multiple arterial thromboses of the right lower limb	NO	NO	NO
VITT21	60 years old	No personal or family history of haemorrhage or thrombotic and/or vascular disorders has been reported	Left popliteal venous thrombosis; cerebral venous sinus thrombosis; splanchnic (portal and renal) thrombosis; Pulmonary thromboembolism	/	NO	NO	NO



Graph 1: Total variants (rare and not rare) identified in the six patients with VITT

Sequencing analysis of the 6 patients with VITT resulted in a total number of genetic variants of 140,563. The total number of variants per patient analyzed, split into rare ($MAF \leq 0.01$) and common variants, is shown in the graphs below. The number and the variant type distribution (Graph 1) are similar among each patient analyzed. Among rare variants, ranging from 1,619 to 1,774 in the six patients with VITT, the higher percentage is represented by intronic (ranging from 38.2% to 42.3%), missense (ranging from 28.4% to 30.6%), and synonymous variants (ranging from 18.5% to 21.3%), whereas splice sites (ranging from 3.6% to 5.3%), 5'/'3'-UTR (ranging from 3.9% to 5.1%), nonsense (ranging from 0.4% to 0.7%), and frameshift (ranging from 0.2% to 0.8%) variants are less frequent (Graph 2).



Graph 2: Rare variant type (intronic-missense-synonymous-UTR-splicing-frameshift-nonsense) distribution among patients with VITT.

At first, we chose to focus our attention on rare genetic variants identified in the patients, selecting them based on allele frequencies reported in the gnomAD exome and genome databases. Our decision to concentrate - at least in this initial phase of analysis - on rare variants is justified by the rarity of VITT onset following adenoviral vector vaccination. Specifically, the incidence of VITT in individuals over 60 years of age is approximately 1.02 cases per 100,000 vaccine doses, increasing to approximately 1 in every 50,000–75,000 doses in individuals under 60. Genes included in the variant analysis were selected based on their involvement in specific Gene Ontology (GO) biological processes - namely blood coagulation (GO:0007596), fibrinolysis (GO:0042730), platelet activation (GO:0030168) and aggregation (GO:0070527), integrin-mediated signaling pathways (GO:0007229), and the inflammatory response (GO:0006954) - as well as according to the Human Phenotype Ontology term for autoimmune thrombocytopenia (HP:0001973) (Table 20).

Pathway	Pathway ID	Genes
Blood coagulation	GO:0007596	<i>MERTK, SLC7A11, PF4, GP5, ITGB3, DGKA, ANXA5, F7, GAS6, F13B, SERPING1, GNAI3, KLKB1, F11, PROCR, LNPB, PDIA3, HNF4A, F2R, F3, PROC, GNAI2, ADRA2C, PRSS56, CTSG, SRF, CD59, ADRA2A, ADRA2B, FLNA, CD9, PLCG2, PIK3CG, KNG1, SERPINA1, SERPINC1, GGCX, GP6, VCL, VWF, F2RL1, FBLN1, DGKK, HBB, PAFAH2, METAP1, CSRP1, ANO6, SLC6A4, JMJD1C, CD36, GNAQ, TSPAN32, GP1BB, PLEK, CLIC1, GP9, PLAUR, ILK, RAP2B, HRG, PABPC4, CYP4F11, WNT3A, MYL9, DGKZ, ITPK1, RAB27A, CYP4F2, FGL1, MMRN1, TREML1, HPS6, UBASH3B, PDPN, P2RX1, STXBP1, STXBP3, SHH, LCK, PDGFRA, TFPI2, ACTG1, SYK, DGKH, SCUBE1, F2RL2, HSPB1, ANXA8, MYH9, ENTPD1, FZD6, P2RY12, GPIBA, MAPK14, WAS, F2RL3, GNAS, DGKI, F5, DGKD, SERPIND1, COL3A1, HPS5, VKORC1, CD40LG, APOH, SLC4A1, ADAMTS13, PLSCR1, PDIA2, SRC, CPB2, DGKG, TYRO3, PEAR1, PLAUR, F12, F10, F9, F2, MYL12A, HPS4, PTPN6, PIK3CB, BLOC1S4, FGG, FGB, FGA, ACTB, AXL, PAPSS2, BLOC1S6, VAV3, LMAN1, TFPI, DTNBP1, ITGA2, P2RY1, HGFAC, PIK3CA, ENTPD2, ENPP4, VAV2, C4BPB, SERPINE2, PPIA, MPIG6B, DGKB, GATA1, C1GALT1C1, CD40, PROS1, THBD, AK3, SAA1, SERPINA10, PROZ, BLOC1S3, DGKE, PLG, PLAT, COMP, ADORA2A, TUBB1, FIBP, DGKQ, F13A1, F8, TLN1, AP3B1, IL6, VAV1, FERMT3</i>
Fibrinolysis	GO:0042730	<i>KRT1, SERPING1, SERPINB2, KLKB1, SERPINF2, HRG, GPIBA, ANXA2, CPB2, PLAUR, F12, F2, FGG, FGB, FGA, PROS1, SERPINE1, PLG, PLAT</i>
Integrin mediated signaling pathway	GO:0007229	<i>CDH17, ITGB3, PLPP3, ISG15, ITGB2, PTK2B, BCAR1, DOCK1, NRPI, ITGB1BP1, RCC2, ITGA5, HCK, ITGB6, LIMS2, CEACAM1, ITGA4, ADAM10, ITGAE, ITGB5, COL16A1, ITGAM, ITGA8, ZNF304, ITGA9, ITGAV, ZYX, PTN, CD47, CTNNA1, TSPAN32, PLEK, ITGA7, CUL3, ADAM9, THY1, ILK, FGR, ITGAD, DST, ITGB8, ITGB7, ITGA3, FUT8, ITGA2B, MADCAM1, ADAM15, SYK, PRKD1, MYH9, FYB2, LAT, ERBIN, LAMA5, COL3A1, FERMT1, CDC42, CD40LG, FN1, ADAMTS13, FYB1, SRC, PRAM1, ITGA11, ABL1, APOA1, ITGA1, NEDD9, VAV3, CCM2, ITGBL1, ITGA2, ITGAX, ITGAL, TEC, ADAM11, ITGB1, MPIG6B, ANGPTL3, FERMT2, TXK, ITGA10, NME2, ADAMTS1, CCN2, SEMA7A, PTPRA, PTPN11, TLN1, ITGB4, ITGA6, VAV1, FERMT3, PTK2</i>
Autoimmune thrombocytopenia	HP:0001973	<i>ACP5, ADA, ARHGEF1, CASP10, CD19, CD81, CIITA, CR2, CTLA4, CTNBL1, FAS, FASLG, FOXP3, GALC, ICOS, IL7R, IRF2BP2, ITK, KDM6A, KMT2D, KRAS, LAT, LRBA, MAGT1, MS4A1, NFKB1, NFKB2, NRAS, PNP, PRKCD, RASGRP1, RFX5, RFXANK, RFXAP, SASH3, SMPD1, SOCS1, STAT1, STAT3, TLR7, TNFRSF13B, TNFRSF13C, TNFSF12, TOM1, TPP2, ZAP70</i>
Platelet aggregation	GO:0070527	<i>SLC7A11, ITGB3, PDIA3, FLNA, PIK3CG, VCL, HBB, METAP1, CSRP1, SLC6A4, TSPAN32, PLEK, CLIC1, ILK, RAP2B, WNT3A, MYL9, FGL1, UBASH3B, STXBP1, STXBP3, PDGFRA, ACTG1, HSPB1, MYH9, P2RY12, F2RL3, GNAS, PDIA2, TYRO3, PEAR1, MYL12A, PTPN6, PIK3CB, BLOC1S4, FGG, FGB, FGA, ACTB, PPIA, GATA1, COMP, TUBB1, FIBP, TLN1, FERMT3</i>
Platelet activation	GO:0030168	<i>MERTK, SLC7A11, PF4, ITGB3, DGKA, GNAI3, PDIA3, F2R, ADRA2C, CTSG, SRF, ADRA2A, ADRA2B, FLNA, CD9, PLCG2, PIK3CG, GP6, VCL, VWF, DGKK, HBB, METAP1, CSRP1, SLC6A4, TSPAN32, GP1BB, PLEK, CLIC1, ILK, RAP2B, HRG, WNT3A, MYL9, DGKZ, FGL1, TREML1, UBASH3B, PDPN, P2RX1, STXBP1, STXBP3, LCK, PDGFRA, ACTG1, SYK, DGKH, F2RL2, HSPB1, MYH9, FZD6, P2RY12, GPIBA,</i>

		MAPK14, F2RL3, GNAS, DGKI, DGKD, COL3A1, CD40LG, ADAMTS13, PLSCR1, PDIA2, SRC, DGKG, TYRO3, PEAR1, F2, MYL12A, PTPN6, PIK3CB, BLOC1S4, FGG, FGB, FGA, ACTB, AXL, VAV3, P2RY1, PIK3CA, ENTPD2, VAV2, SERPINE2, PPIA, DGKB, GATA1, C1GALT1C1, CD40, SAA1, BLOC1S3, DGKE, COMP, TUBB1, FIBP, DGKQ, TLN1, IL6, VAV1, FERMT3
Inflammatory response	GO:0006954	LBP, ATAT1, IL17F, ACER3, WDR83, CCL3L1, SMO, MYD88, FOS, TIRAP, S100A9, POLB, SELE, CCL22, CMKLR1, KLRG1, LTB4R, CHIA, TICAM1, IL17D, CAMK1D, CUL3, TRIM55, CX3CL1, HLA-DRB1, P2RX1, LOXL3, TOLLIP, FFAR4, IDO1, TNIP1, LGALS9, CCL24, CCRL2, ODAM, GPER1, S1PR3, HMGB2, CCL19, BLNK, IKBKG, MDK, CXCR6, SNAP23, WNT5A, PYDC2, ALOX15, PLGRKT, NLRP10, NOTCH1, PTX3, CXCL11, ALOX5, GGT5, CHI3L1, OLR1, RIPK1, SCNN1B, NRROS, REG3G, PIK3CD, ATRN, CCR6, CCR5, CYBB, CCR4, CCR3, CCL11, HAVCR2, NINJ1, IL20RB, DPEP1, SCN9A, SYK, LY96, SCYL3, TLR1, TNF, SIRT2, RAC1, IFNA2, CNTF, PRKD1, GRN, AGER, SCUBE1, BMPR1B, TFR2, PLA2G4C, RELB, NUPR1, IL34, CERS6, CYP26B1, BPGM, PTGER1, JAM3, CNR2, IL36G, IL36B, IL37, TREM2, PLA2G10, CCL16, HDAC4, TLR3, CCL25, NR3C1, PLA2G3, DHX9, TSPAN2, NLRP3, HSPG2, TNFRSF1A, IL22, NR1H4, HNRNPA0, PTGER4, NLRP2, XCR1, HAMP, TLR9, APOL2, APOL3, THBS1, TREX1, BMP2, TNFAIP6, HK1, LYN, IL5RA, VCAM1, MIF, TFRC, CXCL10, PPBP, CD40LG, ORM1, FN1, C1QA, APCS, CRP, GPR33, HMGB1, IL4R, CRHBP, PLSCR1, CHUK, ORM2, IL13, TLR7, CD68, TMEM258, SPHK1, UNC13D, FPR3, CSF1, HMOX1, TSPAN18, IL1RL1, IL10RB, CXCL1, KDM6B, FKRP, ADAM8, ADCY1, TLR10, PLA2G2A, MEFV, FFAR2, CXCL3, CXCL2, PTGDR, NFKB1, FOLR2, HYAL3, CCL26, PLAA, DAB2IP, C5AR2, ACKR1, CAMK4, IL17A, IL23R, CSF1R, PRCP, SIGIRR, LAT, PSTPIP1, TLR6, RPS6KA4, MMP25, JMJD7-PLA2G4B, IL17RC, EIF2AK1, HRH4, TXNIP, RIPK2, CXCR3, TMIGD3, ADORA3, CASP4, TUSC2, WNK4, FUT7, CXCL13, CHST1, TYROBP, F12, HDAC9, HP, F2, IFI16, HIF1A, COL6A1, CCL14, CELA1, EPHA2, TNIP3, AGTR1, MACIR, LIAS, IRGM, EGFR, RPS6KA5, FASN, KIT, PF4V1, BTK, SAA2, SAA1, REG3A, NDST1, LYZ, CD163, SMPDL3B, IL1RL2, LACC1, SIGLEC1, PLD3, TRIM31, CCL20, TAC4, ELF3, SPINK7, UMOD, LY75, CTNBNIP1, TRIM14, NLRP4, FOSL1, FOSL2, NKG7, ECM1, DHRS7B, CXCL5, CCL1, PARP4, CEBPB, UGT1A1, IL10, CLU, CD5L, AHSG, GPR32, RBPJ, IL25, ASS1, SCYL1, FCGR2A, FCGR1A, AOC3, TNFRSF1B, MEP1B, MYLK3, IL17C, IL1RAP, ADORA2A, IL23A, ITGAL, ACVR1, NFE2L2, PRKCQ, NOTCH2, MTOR, TNIP2, IL33, CD2AP, IL17RE, ADGRE2, ITGB1, IRAK2, FCGR3B, PBXIP1, SCN11A, TRAF3IP2, NAIP, AGR2, FUT4, SLC11A1, MFHAS1, CHST2, CX3CR1, SNCA, CCL4L2, MAPT, IL17B, BMP6, TBK1, NFKBID, IL36A, CCR7, CCR1, STK39, REL, FADS2, NFATC4, MAP3K7, C4B, C4A, IL31RA, VNN1, C3AR1, LARGE1, ZEB2, PNMA1, CCL3, CXCL8, CD40, C2CD4A, ZC3H12A, ITIH4, H2BC1, TAC1, ZNF580, PSEN1, FCER1A, CHST4, GNAT2, CYSLTR1, SLAMF8, NOD1, CCL15, GSDMD, PLD4, IL27, NFAM1, MAPKAPK2, SEMA7A, PRDX2, CCL4, CD14, TLR4, IL1R1, S100A12, PLA2G2E, BDKRB1, AP3B1, F8, EXT1, PYCARD, CDO1, TLR5, TLR2, IL18R1, CCR2, IL17RA, ADORA1, CD96, SAA4, NCR3, IKBKB, CSRP3, MAP2K3, PF4, HDAC5, C2CD4B, ADCY8, AIM2, CD44, FFAR3, HYAL1, PJA2, GATA3, AGTR2, CYBA, SLAMF1, KRT16, PLP1, STAB1, JAK2, NOX4, IL5, IL4, S100A8, ITGB2, NLRC4, KLKB1, APP, NLRP1, CD6, IRF5, CLEC7A, BCL6, NFKB2, PLA2G2D, MRGPRX1, B4GALT1, NAGLU, MSMP, ASH1L, IFNGR1, IL9, MAPK9, F2R, F3, LXN, PTAFR, TACR1, CXCR4, HRH1, ANXA1, ADM, GBP5, NFKBIZ, NDP, CXCL9, AFAP1L2, TICAM2, LY86, SELP,

		<i>ITGB6, IL1RN, KDM4D, SERPINF2, VPS54, FCGR3A, HCK, TNFSF4, TCIRG1, PXX, IL2RA, EPO, IL1B, IL1A, IFNG, FOXP3, ELANE, CARD8, F2RL1, CCL21, ACKR2, AIF1, IL18, NFX1, NFATC3, AIMP1, PRDX5, TGFB1, TMED2, P2RX7, LIPA, KCNJ8, CXCL6, SLC18A2, NLRP9, CCL17, PROK2, TNFAIP3, PIK3CG, KNG1, TIMP1, C5, C3, SERPINA3, SERPINA1, PRKCZ, IFNGR2, MGLL, AKT1, TRIL, PTGER2, PTGER3, SMAD1, MBL2, CCL7, CCL8, ABCF1, INS, TNFRSF4, TLR8, CRH, ADGRE5, TBXA2R, C5AR1, CALCA, CCL13, THEMIS2, FCGR2B, CD180, PARK7, PTN, POMGNT1, ANO6, TRPV1, ITGAM, PYDC5, GPR68, FCGR2C, FPR1, XCL1, NLRP6, IL1F10, RARRES2, PTGFR, LRP1, FPR2, SETD4, CXCR2, ACOD1, CCL18, CCL23, NOS2, PTGIR, A2M, NFKBIB, F11R, CD47, NOX1, CD36, IL6R, SCG2, CCL5, CCL2, CARD18, AXL, IL36RN, TAB2, XCL2, MAP3K20, STAT3, ALOX5AP, POMT2, ITCH, NKIRAS2, RELA, RASGRP1, IL18RAP, BDKRB2, NR4A1, IL6, AZU1</i>
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Table 20: Genes for variants analysis selected according to Gene Ontology biological processes or Human Phenotype Ontology

Table 21 lists all the variants identified in the six patients within the previously described biological and functional processes. Specifically, the table includes the patient identifiers, the affected gene with its reference transcript, a description of the identified variants, the *in-silico* tools used to predict the pathogenicity of missense and splicing variants, and the classification of each variant according to ACMG (American College of Medical Genetics and Genomics) guidelines (Richards S. et al., 2015) (in bold are highlighted VUS variants in all patients).

In total, 194 heterozygous variants were identified across the six patients. According to ACMG criteria, 47 of these variants (24.2%) were classified as Variants of Uncertain Significance (VUS), while the remaining ones were considered benign or likely benign.

Specifically, the distribution of VUS was as follows: 4 in patient VITT05, 4 in VITT21, 6 in VITT03, 9 in VITT18, 11 in VITT06, and 13 in VITT02.

No variants were classified as likely pathogenic or pathogenic according to ACMG standards.

Interestingly, although the specific variants differed, both VITT02 and VITT06 harbored a variant in the *FGA* gene, while VITT02 and VITT18 shared a variant in the *SERPINA12* gene and also VITT02 and VITT21 shared a variant in the *JMJD-PLA2G4B* gene.

Table 21: Rare variants identified in VITT patients in the selected genes

Table 21: Rare variants identified in VITT patients in the selected genes					In-Silico Prediction								
Patient	Gene Symbol and Transcript ID	dbSNP ID	Genetic Variant Nomenclature	Minor Allele Frequency	FATHMM	MutationTaster	Provean	Polyphen	Sift	NetGene2	ASSP	SpliceAI	ACGM Variant Classification
VITT02	ITGA2B NM_000419	rs199641871	c.457G>A (p.Ala153Thr)	0.0002339	D	D.C.	N	D	T				VUS
VITT02	ITGAD NM_005353	rs147321998	c.736C>T (p.Arg246Ter)	0.002425	-	-	-	-	-				VUS
VITT02	THBD NM_000361	rs888161210	c.1465G>C (p.Asp489His)	N/A	T	P	N	P.D.	D				VUS
VITT02	FGA NM_000508	rs1257998751	c.1132T>A (p.Ser378Thr)	N/A	T	P	N	B	T				VUS
VITT02	CTNNA1 NM_001903	rs371054484	c.377G>C (p.Arg126Pro)	0.00001548	T	D.C.	D	P.D.	D				VUS
VITT02	PIEZO1 NM_001142864	rs776672249	c.2545C>T (p.Arg849Cys)	0.00001668	T	D.C.	D	D	D				VUS
VITT02	GLCE NM_015554	rs199559391	c.1357A>G (p.Thr453Ala)	0.0001583	T	D.C.	N	D	T				VUS
VITT02	ENPP6 NM_153343	rs147979605	c.541C>G (p.Arg181Gly)	0.00001765	T	D.C.	N	B	T				VUS
VITT02	SERPINA12 NM_173850	rs61758960	c.608T>C (p.Leu203Pro)	0.004	D	D.C.	D	D	D				VUS
VITT02	BMPR1B NM_001256793	rs772708128	c.596_598del (p.Tyr199_Ile200delinsPhe)	0.0000177	-	-	-	-	-				VUS
VITT02	CARD8 NM_001184900	rs369625179	c.1217A>G (p.Tyr406Cys)	0.0000439	T	P	D	P.D.	D				VUS
VITT02	EGFR NM_005228	rs371229748	c.3244A>T (p.Ile1082Leu)	0.0000266	T	P	N,D	B	T				VUS
VITT02	JMJD7-PLA2G4B NM_005090	rs758353242	c.1813C>G (p.Leu605Val)	0.00013	T	P	N	D	P.D.				VUS
VITT02	SERPINB4 NM_002974	rs137899169	c.917A>G (p.Asn306Ser)	0.0003412	D	P	N	B	T				LB
VITT02	LRBA NM_006726	rs143625481	c.787C>G (p.Leu263Val)	0.0009014	T	D.C.	N	D	T				LB
VITT02	FGB NM_005141	rs201909029	c.534G>C (p.Lys178Asn)	0.0005977	D	D.C.	N	B	T				LB
VITT02	HPS5 NM_181507	rs61755718	c.3045G>A (p.Met1015Ile)	0.00798	T	P	N	B	T				LB
VITT02	PTGER3 NM_198718	rs568967213	c.1185del(p.Asn395LysfsTer)	0.008279	-	-	-	-	-				LB
VITT02	FANCL NM_001114636	rs55849827	c.112C>T (p.Leu38Phe)	0.004304	T	D.C.	N	D	D				LB
VITT02	SLX4 NM_032444	rs150712805	c.2305G>C (p.Glu769Gln)	0.001644	T	P	N	B	T				LB
VITT02	PRDM16 NM_022114	rs199968728	c.142G>A (p.Val48Met)	0.0004263	T	P	N	B	T				LB
VITT02	TNFSF12 NM_172089.4	rs768061768	c.43G>A (p.Glu15Lys)	0.0002358	D	P	N	B	D				LB

VITT02	ITGA9 NM_002207	rs61751189	c.1691G>A (p.Arg564Gln)	0.005522	T	D.C.	N	P.D.	D							LB
VITT02	ITGA11 NM_001004439	rs568152331	c.3359G>A (p.Arg1120His)	0.0002078	T	P	N	B	T							LB
VITT02	MASTL NM_001172303	rs36121140	c.1010C>A (p.Thr337Lys)	0.001710	T	P	N	B	T							B
VITT02	PLA2G4A NM_024420	rs28395831	c.1909A>G (p.Ile637Val)	0.009599	T	D.C.	N	B	T							B
VITT02	BDKRB1 NM_000710	rs143823168	c.571C>G (p.Leu191Val)	0.00318	T	P	N	D	D							LB
VITT02	FASN NM_004104	rs2228306	c.6014T>C (p.Val2005Ala)	0.00206	T	D.C.	N	B	T							B
VITT02	FASN NM_004104	rs9898060	c.6012-12G>C	0.00204	-	-	-	-	-	NEG	NEG	NEG				B
VITT02	FASN NM_004104	rs17848927	c.1493-16G>A	0.00222	-	-	-	-	-	NEG	NEG	NEG				B
VITT02	TRIM31 NM_007028	rs201869087	c.513+3A>G	0.00387	-	-	-	-	-	NEG	NEG	DONOR LOSS				B
VITT02	ALOX15 NM_001140	rs41432647	c.1849C>T (p.Pro617Ser)	0.00437	T	P	N	B	T							LB
VITT02	APOL3 NM_145640	rs142057520	c.682G>A (p.Ala228Thr)	0.00353	T	P	N	P.D.	T							LB
VITT02	CCL4L2 ENST00000394465	rs1597769113	c.208G>A (p.Gly70Ser)	0.00594	-	-	-	-	-							LB
VITT02	FASN NM_004104	rs368725295	c.6163+8C>G	0.0000629	-	-	-	-	-	NEG	NEG	NEG				LB
VITT02	PTGER3 NM_198718	rs568967213	c.1185del (p.Asn395LysfsTer9)	0.00834	-	-	-	-	-							LB
VITT02	SETD4 NM_017438	rs140814445	c.1212A>T (p.Lys404Asn)	0.00475	T	P	N	B	T							LB
VITT02	HSPG2 NM_005529	rs141280063	c.12874G>A (p.Glu4292Lys)	0.000967	T	D.C.	N,D	P.D.	T							LB
VITT03	EPB41 NM_001166005	rs150835844	c.1228G>T (p.Asp410Tyr)	0.0002479	D	D.C.	D	D	D							VUS
VITT03	PDIA6 NM_005742	rs768426261	c.256C>T (p.His86Tyr)	0.00007778	T	D.C.	D	D	T							VUS
VITT03	DST NM_001144769	N/A	c.901C>T (p.Pro301Ser)	N/A	D	D.C.	D	-	D							VUS
VITT03	SMPD1 NM_000543	rs142215226	c.340G>A (p.Val114Met)	0.001060	D	D.C.	N	-	D							VUS
VITT03	AKT1 NM_001014431	rs768025881	c.349_351dup (p.Glu117dup)	0.0000088	-	-	-	-	-							VUS
VITT03	NOTCH2 NM_024408	rs765404709	c.7114G>C (p.Ala2372Pro)	0.00000879	D	D	N	B	T							VUS
VITT03	PTAFR NM_000952	rs138629813	c.341A>G (p.Asn114Ser)	0.003554	T	D.C.	N	P.D.	D							LB
VITT03	SLX4 NM_032444	rs145137472	c.2681T>G (p.Val894Gly)	0.0006039	T	P	N	B	T							LB
VITT03	PRDM16 NM_022114	rs371654192	c.2468G>C (p.Arg823Pro)	0.004107	T	D.C.	D	D	D							LB
VITT03	RPL5 NM_000969	rs11540832	c.629A>G (p.Tyr210Cys)	0.008106	T	D.C.	N	B	T							LB
VITT03	F13B NM_001994	rs17514281	c.1025T>C (p.Ile342Thr)	0.01049	T	P	D	P.D.	D							LB
VITT03	HPSE	rs61755719	c.707T>G (p.Phe236Cys)	0.007042	T	P	N	B	T							LB

[illegible]

VITT05	PIEZO1 NM_001142864	rs530486445	c.1495G>A (p.Val499Ile)	0.0009186	D	P	N	B	T								LB
VITT05	ITGB4 NM_000213	rs149659118	c.2009G>A (p.Arg670His)	0.001433	D	P	N	B	T								LB
VITT05	PRAM1 NM_032152	rs138042924	c.1159T>C (p.Ser387Pro)	0.004513	T	P	D	-	D								LB
VITT05	HABP2 NM_004132	rs78201625	c.364C>T (p.Arg122Trp)	0.003043	T	P	N	D	T								B
VITT05	IL6 NM_000600	rs2069860	c.485A>T (p.Asp162Val)	0.007710	T	P	D	B	N								B
VITT05	VEGFB NM_003377	rs111555072	c.286C>G (p.Gln96Glu)	0.001883	T	P	N	P.D.	D								B
VITT05	NFKB1 NM_003998	rs4648072	c.1519A>G (p.Met507Val)	0.007642	T	P	N	B	T								B
VITT05	DAB2IP NM_032552.4	rs570101345	c.2948C>T (p.Ala983Val)	0.0000266	T	P	N	B	T								LB
VITT05	GPB1 NM_001098201.3	rs138657567	"c.1119T>G (p.Ser373Arg)	0.00648	T	P	N	B	D								LB
VITT05	CSF1 NM_000757.6	rs2229165	c.1312G>A (p.Gly438Arg)	0.00696	T	P	N	B	T								B
VITT05	MTOR NM_004958.4	rs56412200	c.7367-3T>C	0.000651	-	-	-	-	-	NEG	NEG	NEG					B
VITT05	AGER NM_001206929.2	rs3176931	c.1154G>A (p.Arg385Gln)	0.00189	T	P	N	B	T								LB
VITT05	AKT1 NM_001014431.2	rs138386095	c.288-14C>T	0.000897	-	-	-	-	-	NEG	NEG	NEG					LB
VITT05	CHIA NM_201653.4	rs182022651	c.169G>A (p.Ala57Thr)	0.000415	T	D.C.	D	D	D								LB
VITT05	IL1R1 NM_000877.4	rs56081302	c.487-6A>G	0.00211	-	-	-	-	-	NEG	NEG	NEG					LB
VITT05	IL1RL1 NM_016232.5	rs34210856	c.239C>T (p.Ala80Val)	0.00215	T	P	N	B	T								LB
VITT05	IL1RL1 NM_016232.5	rs34225180	c.526G>A (p.Ala176Thr)	0.00212	T	P	N	B	T								LB
VITT05	NOTCH2 NM_024408.4	rs41313282	c.4238T>A (p.Leu1413His)	0.00515	T	P	D	B	D								LB
VITT05	IL1R1 NM_000877.4	rs34889382	c.604T>C (p.Tyr202His)	0.0021	T	P	N	B	T								LB
VITT05	IL1R1 NM_000877.4	rs34835752	c.1192G>A (p.Gly398Arg)	0.00211	T	P	D	D	D								LB
VITT05	SELE NM_000450.2	rs73041408	c.1165C>T (p.Arg389Cys)	0.0000882	T	P	N,D	P.D.	D								LB
VITT05	TRIM31 NM_007028.5	rs142316069	c.899A>T (p.Asp300Val)	0.000515	T	P	D	P.D.	D								LB
VITT05	CD96 NM_005816.5	rs370809674	c.1250-13T>A	0.0000352	-	-	-	-	-	NEG	NEG	NEG					LB
VITT05	PRDX2 NM_005809.6	rs778577772	c.380+5T>C	0.0000176	-	-	-	-	-	NEG	NEG	NEG					LB
VITT06	FGA NM_000508	N/A	c.2068C>G (p.Gln690Glu)	N/A	D	D.C.	N	B	T								VUS

VITT06	VASP NM_003370	rs202205375	c.1130G>C (p.Arg377Pro)	0.00001771	T	D.C.	N	D	D				VUS
VITT06	TBXAS1 NM_001166253	rs759354516	c.1673T>C (p.Leu558Pro)	0.00005285	T	D.C.	D	D	T				VUS
VITT06	PIK3CA NM_006218.4	N/A	c.834G>A (p.Met278Ile)	N/A	T	D.C.	N	B	T				VUS
VITT06	LIMS2 NM_017980	rs770469332	c.254G>A (p.Arg85Gln)	0.0000177	D	D.C.	D	P.D.	D				VUS
VITT06	VCL NM_014000.3	rs754046223	c.1298G>A (p.Arg433His)	N/A	T	D.C.	N	P.D.	D				VUS
VITT06	ENTPD1 NM_001164178	rs142591047	c.1226T>C (p.Ile409Thr)	0.000007742	T	P	D	B	T				VUS
VITT06	FFAR2 NM_005306.3	N/A	c.913G>A (p.Asp305Asn)	N/A	T	P	N	B	T				VUS
VITT06	NDST1 NM_001543.5	rs201660056	c.2426C>T (p.Ala809Val)	0.0000439	D	D.C	N	B	D				VUS
VITT06	NLRP2 NM_001174081.3	rs140225599	c.398-2A>T	0.000651	-	-	-	-	-	Suggestive of splicing alteration	Suggestive of splicing alteration	Suggestive of splicing alteration	VUS
VITT06	RPS6KA4 NM_003942.3	rs138221123	c.427G>A (p.Gly143Ser)	0.000571	T	D.C	D	D	D				VUS
VITT06	GAS6 NM_000820	rs770488795	c.1592G>A (p.Arg531His)	0.0001482	T	P	N	B	T				LB
VITT06	SERPINA10 NM_016186	rs2232708	c.811G>A (p.Gly271Ser)	0.006813	D	P	N	B	T				LB
VITT06	LRRFIP1 NM_001137552	rs3739041	c.1897A>G (p.Lys633Glu)	0.003172	T	P	N	B	T				LB
VITT06	LRRFIP1 NM_001137552	rs3739040	c.1934C>T (p.Pro645Leu)	0.003196	T	P	N	P.D.	T				LB
VITT06	SLX4 NM_032444	rs147492092	c.2654C>T (p.Pro885Leu)	0.0004568	T	P	D	P.D.	D				LB
VITT06	UNC13D NM_199242	rs118049905	c.2896C>T (p.Arg966Trp)	0.007380	T	D.C.	D	D	D				LB
VITT06	COL5A1 NM_001278074	rs761079177	c.805G>A (p.Glu269Lys)	0.0001084	D	D.C.	N	B	T				LB
VITT06	ENTPD2 NM_203468	rs374256982	c.980G>A (p.Arg327Gln)	0.00003122	T	P	N	B	T				LB
VITT06	PIEZO1 NM_001142864	rs564220264	c.4526G>A (p.Ser1509Asn)	0.00003554	T	P	N	B	T				LB
VITT06	RFX5 NM_000449.4	rs1374291718	c.1283A>G (p.Lys428Arg)	N/A	T	D.C.	N	D	D				LB
VITT06	PPAN-P2RY11 NM_001040664	rs199800140	c.1018C>T (p.Arg340Trp)	0.0003402	T	D.C.	D	D	D				LB
VITT06	PPAN-P2RY11 NM_001040664	rs776329867	c.1730C>T (p.Ala577Val)	0.000009221	T	P	N	B	T				LB
VITT06	SERPINA10 NM_016186.2	rs2232698	c.262C>T (p.Arg88Ter)	0.006544	-	-	-	-	-				B
VITT06	LRRFIP1 NM_001137552	rs3739041	c.1897A>G (p.Lys633Glu)	0.003172	T	P	N	B	T				B
VITT06	LRRFIP1 NM_001137552	rs3739040	c.1934C>T (p.Pro645Leu)	0.003196	T	P	N	B	T				B

VITT06	KMT2D NM_003482	rs189888707	c.7670C>T (p.Pro2557Leu)	0.006668	T	D.C.	D	B	D				B
VITT06	SELE NM_000450.2	rs79478039	c.975C>A (p.Phe325Leu)	0.00423	T	P	D	B	T				LB
VITT06	ABCF1 NM_001025091.2	rs6902544	c.592A>G (p.Asn198Asp)	0.00822	T	P	N	B	T				LB
VITT06	KDM4D NM_018039.3	rs76057256	c.1070A>G (p.Glu357Gly)	0.00202	T	P	D	D	D				LB
VITT06	NLRP9 NM_176820.4	rs139574367	c.2331-17G>A	0.00385	-	-	-	-	-	NEG	NEG	NEG	LB
VITT06	LGAL59 NM_009587.3	rs61736863	c.675G>T (p.Met225Ile)	0.00449	T	P	N	B	T				LB
VITT06	UNC13D NM_199242.3	rs118049905	c.2896C>T (p.Arg966Trp)	0.00763	T	D.C	D	D	D				LB
VITT18	GP6 NM_001083899	rs779660245	c.830G>A (p.Arg277Gln)	0.00005 (ALFA_EU)	T	P	D	D	D				VUS
VITT18	SERPINA12 NM_173850	rs146053420	c.1062C>G (p.His354Gln)	0.0008485	D	D.C.	D	D	D				VUS
VITT18	MFSD2B NM_001346880	rs138903557	c.1000C>G (p.Pro334Ala)	0.003837	D	D.C.	D	D	D				VUS
VITT18	NID2 NM_007361	N/A	c.2353A>G (p.Thr785Ala)	N/A	D	P	D	B	D				VUS
VITT18	COLGALT1 NM_024656	rs764429704	c.487C>G (p.Leu163Val)	0.00002324	D	D.C.	N	P.D.	D				VUS
VITT18	WDFY4 NM_020945	rs765608663	c.193C>T (p.Arg65Cys)	0.0001555	T	P	D	D	D				VUS
VITT18	SAMD9L NM_152703	rs776780720	c.1076G>A (p.Arg359Gln)	0.00001765	T	P	N	D	D				VUS
VITT18	BCL6 NM_001706	rs1234897185	c.592T>C (p.Tyr198His)	0.00000879	T	D.C.	N	D	T				VUS
VITT18	TRPV1 NM_018727	N/A	c.1064A>G (p.Gln355Arg)	N/A	T	D.C.	N	B	T				VUS
VITT18	PAFAH1B3 NM_001145940	rs141406696	c.395G>A (p.Arg132Gln)	0.00005419	T	P	N	B	T				LB
VITT18	NOS3 NM_000603	rs3918234	c.2945A>T (p.Gln982Leu)	0.002383	T	D.C.	D	B	T				LB
VITT18	TYRO3 NM_006293	rs752121582	c.550G>A (p.Ala184Thr)	0.00006219	T	P	N	B	T				LB
VITT18	MMRN1 NM_007351	rs147451161	c.3680G>T (p.Arg1227Leu)	0.002155	D	D.C.	D	D	D				LB
VITT18	FYB NM_001243093	rs201568060	c.440C>T (p.Pro147Leu)	0.001052	T	P	D	B	T				LB
VITT18	MYH9 NM_002473	rs139134727	c.4396C>T (p.Arg1466Trp)	0.003004	T	D.C.	D	D	D				LB
VITT18	ADAM17 NM_003183	rs61754178	c.148A>G (p.Ile50Val)	0.005523	T	D.C.	N	D	T				LB
VITT18	MB21D1/CGAS NM_138441	rs147035222	c.50C>G (p.Ala17Gly)	0.006627	T	P	N	B	T				LB
VITT18	PTGS1 NM_000962	rs5794	c.1441G>A (p.Val481Ile)	0.009401	T	P	N	B	D				LB
VITT18	BRCA2	rs41293475	c.1151C>T (p.Ser384Phe)	0.001188	T	P	N	D	D				LB

VITT18	NM_000059 ALOX15B	rs141549870	c.1574G>A (p.Ser525Asn)	0.0004265	T	P	N	B	T							LB
VITT18	NM_001141 HPS4	rs147435410	c.1396C>T (p.Arg466Cys)	0.002767	T	P	N	B	T							LB
VITT18	NM_152841 DGKG	rs762508343	c.1684G>A (p.Val562Ile)	0.00000882	D	D.C.	N	B	T							LB
VITT18	NM_001346 SCUBE1	rs144373347	c.2449G>A (p.Asp817Asn)	0.004394	T	D.C.	N	B	T							LB
VITT18	NM_173050.5 ITLN1	rs150008974	c.509G>A (p.Arg170His)	0.0002408	T	P	N	B	T							LB
VITT18	NM_017625.3 MB21D1/CGAS	rs147035222	c.50C>G (p.Ala17Gly)	0.006627	T	P	N	B	T							LB
VITT18	NM_13844 LRRFIP1	rs142698219	c.2201C>T (p.Pro734Leu)	0.001077	T	P	N	B	T							LB
VITT18	NM_001137552 ITGAX	rs200451726	c.1798G>A (p.Val600Met)	0.0004921	T	P	N	P.D.	T							LB
VITT18	NM_000887 PLCG2	rs75472618	c.1712A>G (p.Asn571Ser)	0.007328	D	D.C.	N	B	D							B
VITT18	NM_002661.5 C5	rs142459293	c.4204A>G (p.Ile1402Val)	0.000897	T	P	N	B	D							LB
VITT18	NM_001735 CCR5	rs1800940	c.180G>T (p.Arg60Ser)	0.00157	T	P	D	B	D							LB
VITT18	NM_001100168 PYDC2	rs377044348	c.245C>T (p.Thr82Met)	0.0000178	-	P	-	B	-							LB
VITT18	NM_001083308 TXNIP	rs34791738	c.520C>T (p.Pro174Ser)	0.0085	-	-	-	-	-							LB
VITT18	NM_006472 GSDMD	rs138749323	c.746G>A (p.Arg249His)	0.000301	T	P	N	D	T							B
VITT18	NM_024736 POMT2	rs138488805	c.2148-18A>G	0.00608	-	-	-	-	-	NEG	NEG	NEG				B
VITT18	NM_013382 AIMP	rs138106524	c.592C>T (p.Pro198Ser)	0.000607	T	D.C.	D	B	D							LB
VITT18	NM_00114241 FPR2	rs74602258	c.220T>C (p.Phe74Leu)	0.00662	T	D.C.	D	P.D.	D							LB
VITT18	NM_001005738 NLRP9	rs80009430	c.126C>G (p.Ile42Met)	0.00379	T	P	N	D	D							LB
VITT021	NM_176820 LRTM2	rs919752328	c.1046G>A (p.Arg349His)	N/A	T	D.C.	N	D	D							VUS
VITT021	NM_001163926 COL5A1	rs201997623	c.404C>T (p.Ser135Phe)	0.00001763	T	D.C.	D	D	D							VUS
VITT21	NM_001278074 ADAM8	rs368130036	c.923C>T (p.Ala308Val)	0.000121	T	P	D,N,D	P.D.	D							VUS
VITT21	NM_001109.5 JMJD7-PLA2G4B	rs146887953	c.66G>T (p.Glu22Asp)	0.00103	T	P	N	B	T							VUS
VITT21	NM_005090.4 SERPINA10	rs2232708	c.811G>A (p.Gly271Ser)	0.006813	D	P	N	B	T							LB
VITT21	NM_016186 BRCA2	rs80359143	c.8918G>A (p.Arg2973His)	0.00003556	T	P	N	P.D.	D							LB
VITT21	NM_000059 ADRA2C	rs1346646919	c.942_950del	0.004276	-	-	-	-	-							B

DISCUSSION

This thesis addresses for the first time whether rare genetic variants could contribute to the development of VITT, a clinical condition mainly associated with COVID-19 adenoviral vector vaccine administration and its adverse complications through a WES approach.

Due to the rare occurrence of VITT, in this thesis, we focused the attention on rare variants. At a whole analysis of the rare variants identified in the 6 patients with VITT, no significant enrichment of mutated genes in biological/functional pathways common to different analyzed subjects was found. Therefore, we focused on potentially damaging mutations on the basis of ACMG criteria, occurring in selected genes involved in biological and functional processes associated with blood coagulation and fibrinolysis, platelet activation and aggregation, integrin-mediated signaling pathway, inflammation with particular attention to those involved in vascular damage, and autoimmune thrombocytopenia. Actually, selected pathways have been described to contribute to a major push in VITT and in its complication severity (Marcucci R. & Marietta M. 2021- Hwang J. et al., 2021 – Greinacher A. et al., 2009 - Greinacher A. et al., 2017).

From a clinical perspective, patients VITT05 and VITT18 exhibited the most unfavorable outcomes. Specifically, patient VITT05 developed severe venous thrombotic events that, due to subsequent complications, resulted in death. Patient VITT18 experienced serious complications that required multiple surgical procedures, leading to significant disabilities and prolonged rehabilitative therapy.

In addition, for each patient, we identified VUS variants ranging from 4 to 13. However, it is important to note that no correlation was observed between the number of variants identified in a patient and the severity of clinical manifestations, outcome severity, recovery trajectory, or circulating anti-PF4 antibody levels during patient follow-up.

In relation to that last point, and according to our study (Lotti et al., 2024), follow-up data showed that in 50% of the ten analyzed patients (six of them also genetically analyzed), anti-PF4 antibody titers remained elevated for several months, persisting for up to 15 months after the acute event [median follow-up duration: 12.5 months (IQR 12–13.5 months)]. These findings are consistent with previously published reports describing a longer persistence of anti-PF4 antibodies in VITT compared to autoimmune heparin-induced thrombocytopenia (aHIT).

In VITT05, a rare VUS in the *STAB2* gene [c.2065C>G (p.Pro689Ala)] has been classified as harmful by most *in-silico* predictors used. *STAB2* gene encodes stabilin-2, expressed predominantly in the sinusoidal endothelium of the liver and spleen and acting as a systemic scavenger receptor for heparin, chondroitin sulfate, dermatan sulfate, nonglycosaminoglycan,

acetylated low-density lipoprotein, procollagen pro-peptides, and advanced glycation end products (Harris E. N. et al., 2008 – Harris E. N et al., 2009). Previous WES data also showed an association of *STAB2* locus with venous thromboembolic disease (Desch K. C. et al., 2020); really, previous data demonstrated that stabilin-2, beyond its role as a scavenger receptor for the aforementioned components, acts as a clearance receptor for VWF, supporting the connection between stabilin-2 and VWF plasma levels (Desch K. C. et al., 2020). Therefore, it could be hypothesized that *STAB2* genetic variants might affect stabilin-2 cell surface expression by possibly inducing protein misfolding or nonsense-mediated decay, thus influencing molecular pathways stabilin-2 mediated and, in turn, contributing to the development of a prothrombotic phenotype. Indeed, it has been observed a role for stabilin-2 in influencing the thrombus incidence in association with elevated VWF plasma levels, potentially by promoting the interaction of platelets and leukocytes with the vessel wall. There appears to be an inverse relationship between the *STAB2* gene and circulating levels of VWF. Specifically, some studies of *STAB2* knockout mice have shown that the absence of this gene leads to increased VWF levels in the bloodstream, which in turn is linked to a higher risk of thrombosis (Michels A. et al., 2021).

VITT05 patient was also a carrier of another rare variant in the *CD47* gene [c.311A>G (p.Asp104Gly)], encoding the leukocyte surface antigen *CD47*, for which a variable prediction of pathogenicity by *in-silico* tools was evidenced. The contribution of the *CD47* gene might be considered critical, as well; really, the leukocyte surface antigen *CD47* plays a role as a receptor for thrombospondin, a secreted matricellular protein observed to be upregulated in vascular cells after injury as well as in chronic disease, and modulator of integrin signaling. Indeed, the CD47-TSP1 binding has been previously shown to influence thrombus formation leading events, including the adhesion of platelets to the endothelial wall, nitric oxide/cGMP (cyclic guanosine monophosphate) signaling induction, platelet activation, and aggregation (Roberts D.D et al., 2012 – Rogerts N. M. et al., 2014 – Jeanne A., et al 2020). VITT05 also exhibited two further VUS in genes involved in inflammation pathways. A rare variant in *COL6A1* gene [c.2191C>T (p.Arg731Cys)], encoding for alpha-1 chain of collagen VI, for which *in-silico* tools used for pathogenicity prediction established a damaging effect, was detected. Data from the literature showed that the *COL6A1* gene was upregulated in patients with nonemphysematous chronic obstructive pulmonary disease, so supporting the profibrotic mast cell phenotype, through collagen VI deposition (Imamoto T. et al., 2023). Moreover, a further variant was found in the *IL5RA* gene [c.913G >A p.(Asp305Asn)], encoding the interleukin-5 receptor subunit alpha, involved in the survival, differentiation, and chemotaxis of eosinophils, contributing to modulate the innate immune system (Sussman T.A. et al., 2019).

Using the STRING database (Search Tool for the Retrieval of Interacting Genes/Proteins), a widely used bioinformatics resource for exploring protein–protein interactions, we simulated interaction networks involving genes affected by VUS in patient VITT05 [Figure 18]. Notably, the analysis suggests a potential relationship between the genes *STAB2* and *CD47*. According to STRING, these genes are co-expressed and may be functionally associated, supporting the hypothesis of a shared or converging biological pathway.

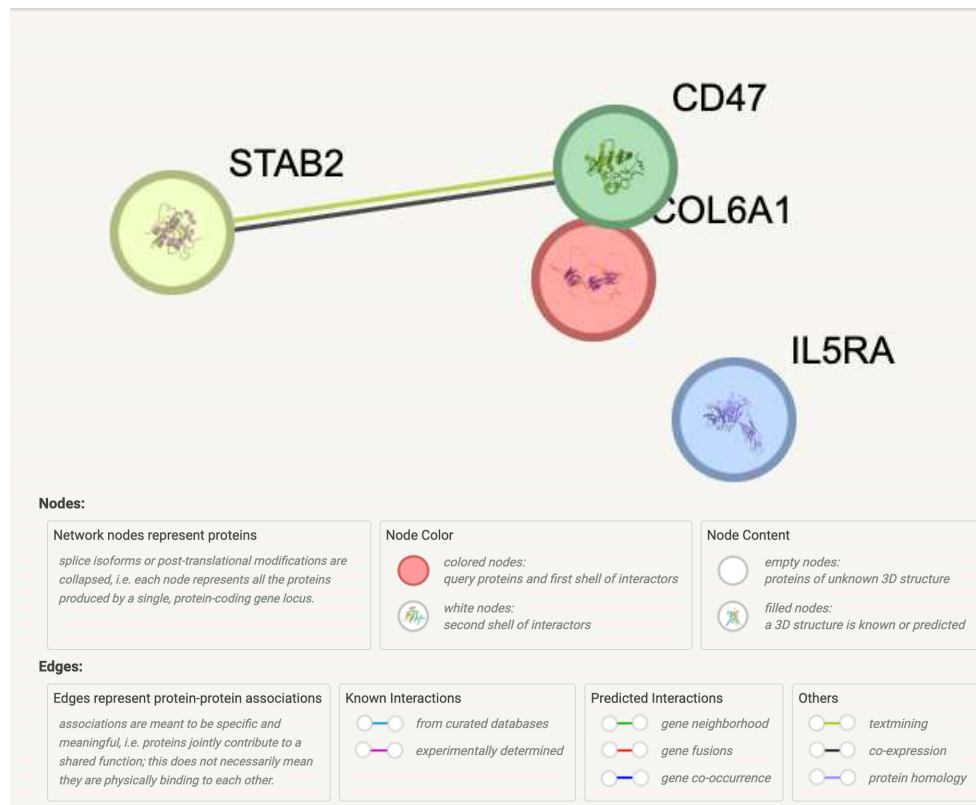


Figure 18: Network of interactions among genes carrying VUS (Variants of Uncertain Significance) in patient VITT05, along with a corresponding legend

VITT18 is a patient with a severe disability as a consequence of cerebral stroke with intraparenchymal hemorrhage. Interestingly, this patient carries a missense variant [c. 830G>A (p.Arg277Gln)] in *GP6* gene encoding for platelet glycoprotein VI (GPVI), a collagen receptor involved in collagen-induced platelet adhesion and activation, contributing to platelet procoagulant activity and thrombin and fibrin formation. The GPVI represents a receptor expressed on platelets and megakaryocytes and acts as a receptor for collagen as well as several plasma and vascular proteins (i.e., laminin, fibronectin, and galectin-3). Data from the literature suggested the contribution of platelet GPVI in influencing venous thrombotic complications; really GPVI might sustain the thrombo-inflammatory status by inducing neutrophil granular release. Moreover, it has been suggested the role of a GPVI-thrombin-fibrin feedforward loop supporting the thrombus formation (Perrella G. et al., 2021). Apart from the *GP6* gene variant,

VITT18 patient also carries two rare missense variants in *SERPINA12* and *MFSD2B* genes [c.1062C>G (p.His354Gln) and c.1000C>G (p.Pro334Ala)], both classified as damaging by all *in-silico* prediction tools. *SERPINA12* gene product, vaspin, is a member of the large family of serine protease inhibitors and has been observed to inhibit target protease kallikrein 7 (KLK7), the process accelerated by the presence of heparin (Oertwig K. et al., 2017). Actually, it has been evidenced that serum vaspin is an independent prognostic marker of major adverse cardiac events (cardiovascular death, recurrent acute myocardial infarction (AMI), or hospitalization for heart failure) in AMI patients. In particular, low vaspin levels have been found to be associated with increased inflammation in AMI patients (Zhou X. et al., 2019).

As concerns *MFSD2B*, it encodes the sphingosine-1-phosphate transporter, able to mediate the export of sphingosine-1-phosphate in red blood cells and platelets (Vu T. M. et al., 2017). A mouse model highlighted that *Mfsd2b* knock-out could influence platelet intrinsic functions, thus supporting its possible role in limiting thrombus formation (Chandrasekaran M. et al., 2021). In particular, the mouse model suggested that sphingosine-1-phosphate signaling could be involved in platelet biogenesis. *Mfsd2b* has been observed to be required for sphingosine-1-phosphate release in resting and activated platelets, really *Mfsd2b* knock-out is associated with a reduced platelet intrinsic function and, in turn, a reduced thrombosis in mice. Therefore, gain-of-function variants in the *MFSD2B* gene might contribute to a prothrombotic phenotype. VITT18 phenotype might be also supported by the presence of two further VUS: a variant in the *BCL6* gene, encoding a transcriptional repressor, able to influence T cell proliferative capacity and fate, and a variant in *TRPV1* gene, encoding a ligand-activated nonselective calcium permeant cation channel involved in the detection of noxious chemical and thermal stimuli. Data from mouse models reported that *Bcl6* expression was reduced in the presence of mir-155 (microRNA 155) deficiency in advanced atherosclerosis, supporting its involvement in the modulation of the atherosclerotic process (Wei Y. et al., 2015). Moreover, *BCL6* has been observed to be involved in live-attenuated influenza vaccine (LAIV) - induced T follicular helper cell differentiation, influencing the antibody response (Aljurayyan A. et al., 2018). As concerns the *TRPV1* gene, it has been shown that brain channels are activated by ischemic stroke, causing neurological and motor deficits as well as infarction after brain ischemia (Miyahara J. et al., 2015). Moreover, it has been reported that *TRPV1* is present in platelets, thus representing a substrate for platelet activation by inflammatory mediators in atherosclerotic plaques (Arper A.G. et al., 2009).

In VITT02 patient, in whom both venous and arterial thrombosis occurred, a VUS with all damaging predictors of pathogenicity [c.608T>C (p.Leu203Pro)] was found in the *SERPINA12* gene. In addition, in VITT02 patient, there has been evidence of the presence of VUS in genes involved in two strictly related biological processes: (cell-matrix adhesion [*ITGA2B*, *ITGAD*, and

FGA, gene ontology term GO:0007160] and blood coagulation [*THBD* and *FGA*, GO:0072377]). Interestingly, VITT02 exhibited the presence of two variants in genes encoding for integrin family members, such as *ITGA2B*, encoding for the integrin alpha-IIb and associated with Glanzmann thrombasthenia (OMIM 273800), and *ITGAD*, encoding for the integrin alpha-D. Altogether, these loci might contribute to explain the thrombotic profile. Among other variants identified, two have not been previously described in the general population; the first one is a rare missense variant (c.1465G>C) in *THBD* gene, encoding for thrombomodulin, an endothelial-specific type I membrane receptor involved in the regulation of the coagulation process, determining the substitution of the Aspartic Acid at position 489 with an Histidine (p.Asp489His) located in the extracellular region in the serine/threonine-rich domain, a binding side for alpha-L/beta-2 and alpha-M/beta-2 integrin (Kawamoto E. et al., 2016); the second one is a rare missense variant [c.1132T>A (p.Ser378Thr)] in the *FGA* gene, encoding for the fibrinogen α -chain. As concerns the *THBD* variant, the serine/threonine-rich domain represents a critical domain for protein anticoagulant activity, and data concerning other variants previously identified in the same domain possibly support its role. Further VUSs have been detected in the *PIEZO1* gene, encoding the piezo-type mechanosensitive ion channel component 1, which was shown to be a key modulator of abnormal platelet activation and thrombosis under hypertension (Zhao W. et al., 2021), and *GLCE* gene, encoding the D-glucuronyl C5-epimerase, involved in heparan sulfate/heparin biosynthesis (Li J.P. 2010). In VITT02 patient, uncertain significant rare variants have also been detected in the *CARD8* and *EGFR* genes, both involved in inflammation pathways. *CARD8* protein represents an inflammasome sensor, mediating the activation of the inflammasome according to pathogen-associated signals response. In particular, it has been reported that *CARD8* participates in the regulation of cytokines and chemokines expression in endothelial cells and atherosclerotic lesions (Paramel G. V. et al., 2021). As concerns the *EGFR* gene, it encodes a receptor tyrosine kinase binding ligand of the *EGF* family. Data from RNA sequencing analysis, performed in glioblastoma tissue specimens according to the thrombotic phenotype, showed a higher *EGFR* expression in the group with many thrombi with respect to that with few thrombi (Furuta T. et al., 2023).

In patient VITT02, as shown in the figure below (Figure 19) generated using the STRING database, several correlations are observed among genes carrying VUS. In particular, three distinct clusters of interactions emerge. The first cluster includes the genes *FGA*, *THBD*, *ITGA2B*, and *ITGAD* - all of which are involved in the hemostatic process. Specifically, *ITGA2B* and *ITGAD* are part of the integrin signaling pathway (GO:0007229); *FGA* is implicated in multiple pathways, including blood coagulation (GO:0007596), fibrinolysis (GO:0042730), platelet activation (GO:0030168), and platelet aggregation (GO:0070527); and *THBD* is directly involved in the blood coagulation pathway (GO:0007596). According to STRING, the correlations

observed among these genes are supported by known protein–protein interactions and by the fact that they participate in shared biological processes. STRING also reveals two additional gene associations: one between *JMJD7-PLA2G4B* and *ENPP6*, based on curated databases, and another involving *BMPR1B*, *EGF*, and *CTNNA1*, supported by similar functional evidence. Furthermore, as illustrated in the protein structure analysis generated using PyMOL (Figure 20), the proteins encoded by *ITGA2B*, *ITGAD*, *FGA*, and *THBD* are structurally connected, suggesting a coordinated role within the hemostatic cascade. The fact that these proteins interact at both the functional and structural levels supports the hypothesis that potentially deleterious variants in these genes may have contributed collectively to the patient's clinical phenotype.

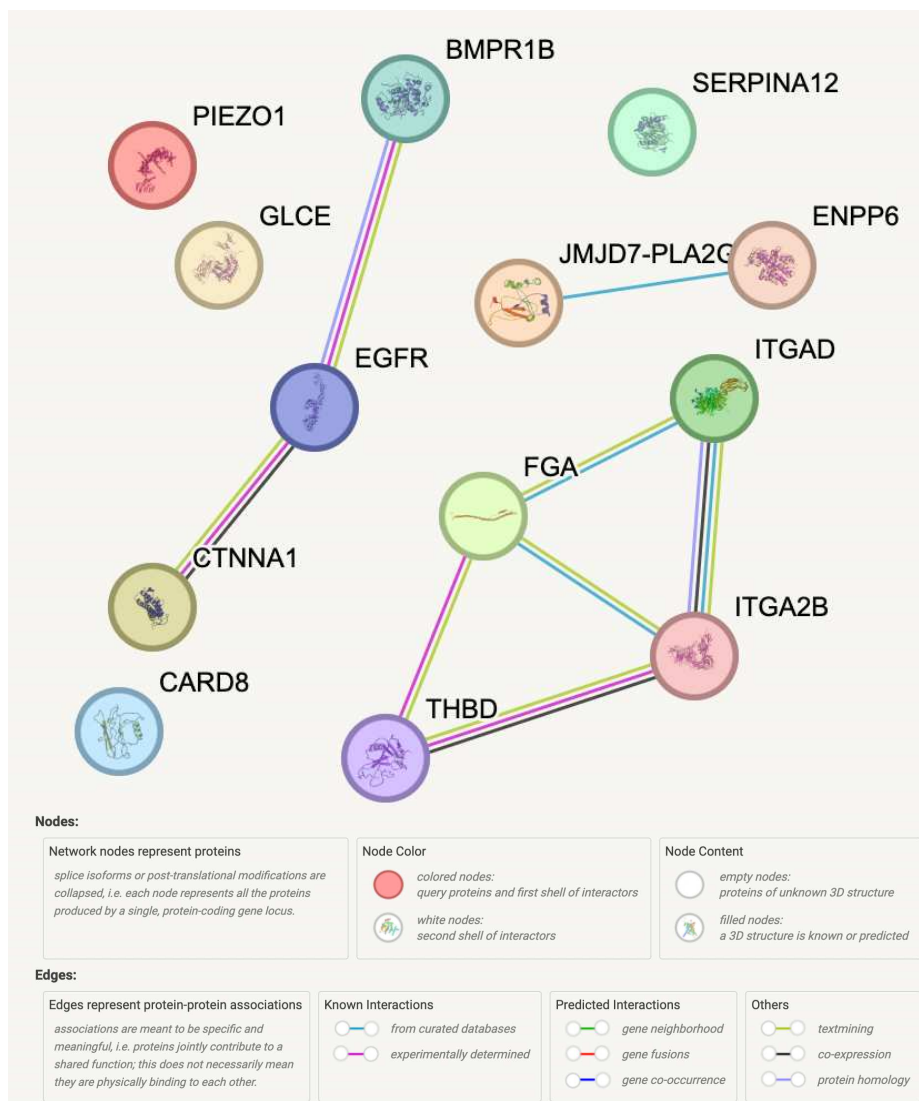


Figure 19: Network of interactions among genes carrying VUS in patient VITT02, along with a corresponding legend

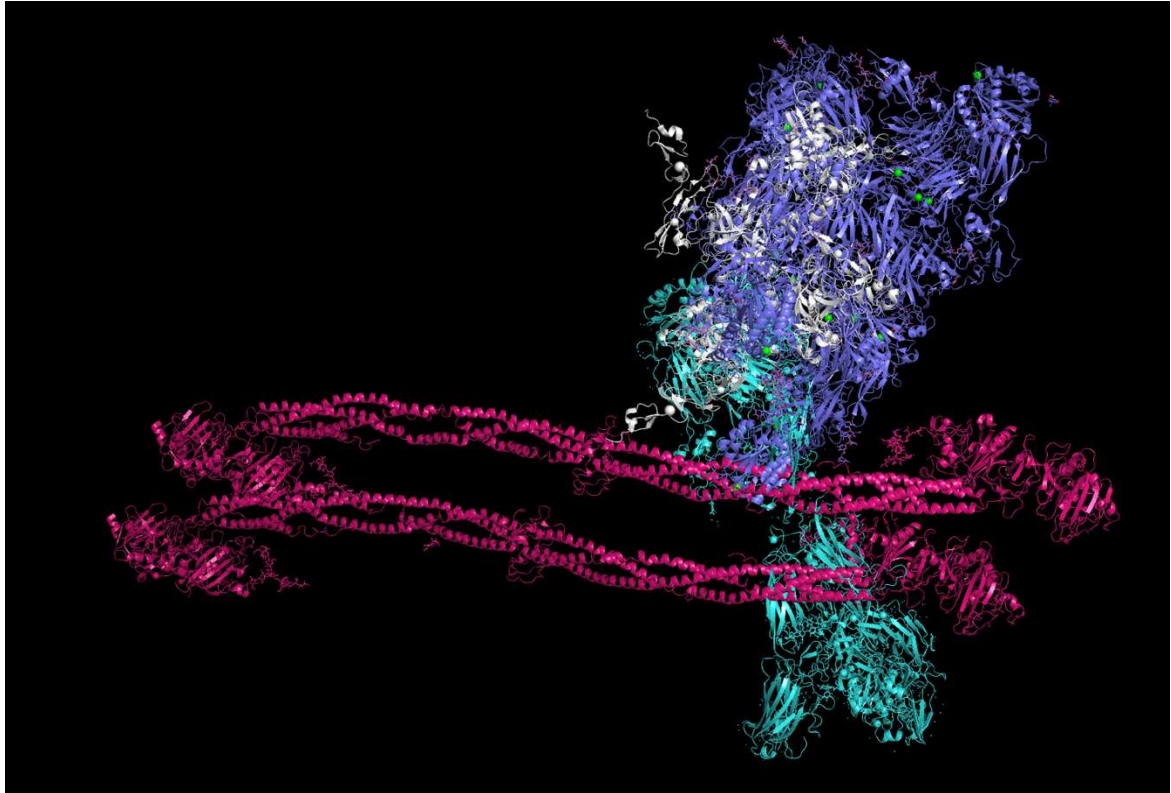


Figure 20: Protein structure visualization generated with PyMOL for the genes carrying variants of uncertain significance (VUS) in patient VITT02. Specifically, the following proteins are highlighted: ITGA2B (PDB ID: 3FCS) in light blue, ITGAD (PDB ID: 5ES4) in violet, the fibrinogen complex - comprising FGA, FGB, and FGG (PDB ID: 3GHG) - in magenta, and the thrombomodulin complex (PDB ID: 1DX5) in white.

Similarly to VITT02, a VUS in the *FGA* gene was also detected in VITT06 patient, also exhibiting both venous thrombosis and stroke. The missense variant identified in this patient [c.2068C>G (p.Gln690Glu)] has not previously been described and exhibited a variable pathogenicity prediction with the *in-silico* tools used, thus supporting the possible contribution of fibrinogen chain alterations to a prothrombotic phenotype. This patient also exhibited VUS in *VASP*, *TBXAS1*, and *ENTPD1* genes. *VASP* gene encodes the vasodilator-stimulated phosphoprotein, actin-associated proteins involved in a range of processes dependent on cytoskeleton remodeling and cell polarity; in particular, it regulates actin dynamics in platelets and plays an important role in regulating platelet activation/aggregation (Benz P. M. et al., 2016 – Aszódi A. et al., 1999). *TBXAS1* gene encodes the thromboxane-A synthase 1, an enzyme responsible for the conversion of prostaglandin H₂ (PGH₂) to thromboxane A₂ (TXA₂), a potent inducer of blood vessel constriction and platelet aggregation (Park S. A et al., 2009). Data from the literature also evidenced the association of thromboxane A synthase 1 gene expression and promotor haplotypes with the risk of large artery-atherosclerosis stroke in the Iranian population (Ramazi S. et al., 2019). As concerns *ENTPD1*, it encodes the ectonucleoside triphosphate diphosphohydrolase 1 observed to be implicated in the prevention of platelet aggregation by hydrolyzing platelet-activating ADP to AMP (Kaczmarek E. et al., 1996). VITT06 patient also exhibited uncertain significance rare variants in *FFAR2*, *NDST1*, and *NLRP2* genes. *FFAR2* gene encodes a G protein-coupled receptor that is activated by a major product of dietary fiber digestion, the short-chain fatty acids (SCFAs), and that plays a role in the regulation of whole-body energy homeostasis and in intestinal immunity. Previous data from the literature reported its role as a potential biomarker to predict AMI occurrence (Ruan J. et al., 2020). *NDST1* encodes a member of the heparan sulfate/heparin GlcNAc N-deacetylase/N-sulfotransferase family. It has been previously reported in mouse models that modification of the heparan sulfate fine structure by *Ndst1* deletion contributes to decrease vascular smooth muscle cells (VSMC) proliferation, as well as vascular remodeling (Adhikari N. et al., 2010). *NDST1* deficiency has also been previously shown to correlate with allergic airway inflammation (Ge X. N. et. al., 2018). The *NLRP2* gene encodes a member of the nucleotide-binding and leucine-rich repeat receptor (NLR) family. It interacts with components of the I κ B kinase (IKK) complex and can regulate both caspase-1 and NF- κ B (nuclear factor kappa-light-chain-enhancer of activated B cells) activity. Previous data from the literature reported higher expression levels both in vitro and in vivo under ischemic stroke conditions (Puleo M. G. et al., 2022).

In the case of patient VITT06, we employed the STRING database to explore potential interactions among the genes harboring variants of uncertain significance. The analysis revealed a notable interaction involving three genes: *VCL*, *VASP*, and *FGA* (Figure 21).

The *FGA* gene is well-characterized for its involvement in key hemostatic processes, including blood coagulation (GO:0007596), fibrinolysis (GO:0042730), platelet aggregation (GO:0070527), and platelet activation (GO:0030168). Similarly, *VCL* (vinculin) has been implicated in pathways associated with blood coagulation, platelet aggregation, and platelet activation, underscoring its potential role in cytoskeletal dynamics and cell-matrix interactions during thrombus formation. In contrast, *LIMS2* is functionally linked to the integrin signaling pathway (GO:0007229), which is critical for mediating cellular adhesion and mechanotransduction, processes that are also relevant to platelet function and vascular integrity. STRING data support the interaction among these genes based on multiple lines of evidence, including genomic proximity, experimentally validated protein-protein interactions, curated pathway annotations, and gene co-expression profiles. Together, these findings suggest a potentially coordinated role for these genes in modulating platelet-related functions and coagulation, which may be clinically relevant in the context of the VUS observed in patient VITT06.

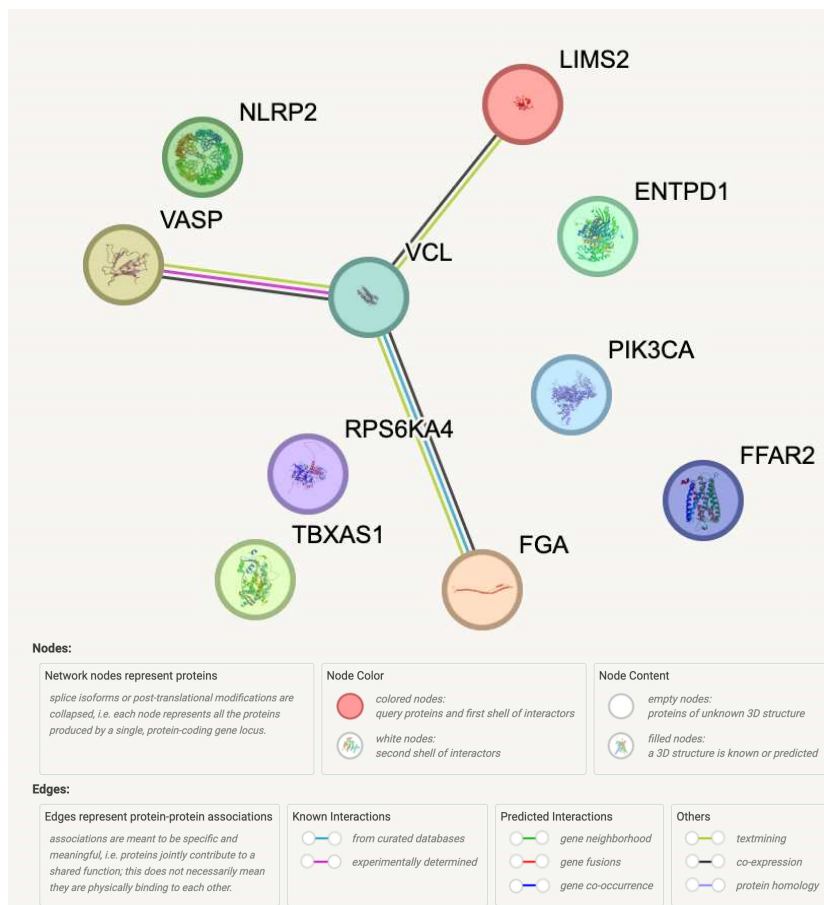


Figure 21: Network of interactions among genes carrying VUS in patient VITT06, along with a corresponding legend

In VITT03, in whom a deep vein thrombosis was observed, a missense VUS [c.1228G>T(p.Asp410Tyr)] classified by all *in-silico* predictors as damaging has been identified in *EPB41* gene, encoding for a structural element of the erythrocyte membrane skeleton, for which gene expression studies evidenced its contribution in the thrombus formation network (Morgan S. et al., 2022). Actually, a decreased erythrocyte membrane flexibility results in an increased red erythrocyte rigidity that might contribute to a thrombogenic potential (Weisel J. W. et al., 2019). Apart from *EPB41*, VITT03 also carries a further missense VUS in the *PDIA6* gene, encoding for the protein disulfide-isomerase A6, that, beyond its role as a molecular chaperone, able to inhibit aggregation of misfolded proteins, also plays a role in platelet aggregation (Jordan P. A. et al., 2004). VITT03 patient also carries uncertain significance rare variants in *AKT1* and *NOTCH2* genes. *AKT1* gene encodes one of the three members of the human AKT serine-threonine protein kinase family. It has been demonstrated to contribute to dendritic cell survival and maturation (Park D. et al., 2006). Moreover, *AKT1* mutations are associated with Proteus syndrome, characterized by an increased risk of thromboembolism (Keppler-Noreuil K. M. et al., 2017). *NOTCH2* gene encodes a member of the Notch family, playing a role in a variety of developmental processes by controlling cell fate decisions. Data from the literature reported that anti-PF4/heparin antibody production is impaired in B-cell-specific Notch2-deficient mice, lacking marginal zone (MZ) B cells (Zheng Y. et al., 2013).

As with other patients we analyzed, we performed a STRING database analysis for VITT03 as well, focusing on the genes harboring variants of uncertain significance. Once again, we identified an interaction among three distinct genes: *SMPD1*, *AKT1*, and *NOTCH2* as we could see in Figure 22. The *SMPD1* gene is associated with the autoimmune thrombocytopenia pathway (HP:0001973), while both *AKT1* and *NOTCH2* are involved in the inflammatory response pathway (GO:0006954). As in previous cases, the interactions identified among these genes are supported by multiple lines of evidence provided by STRING, including frequent co-mentioning in scientific literature (based on text mining), experimentally validated interactions and co-expression data. These findings suggest a potential functional relationship among these genes, possibly contributing to immune-related or inflammatory processes relevant to the patient's phenotype.

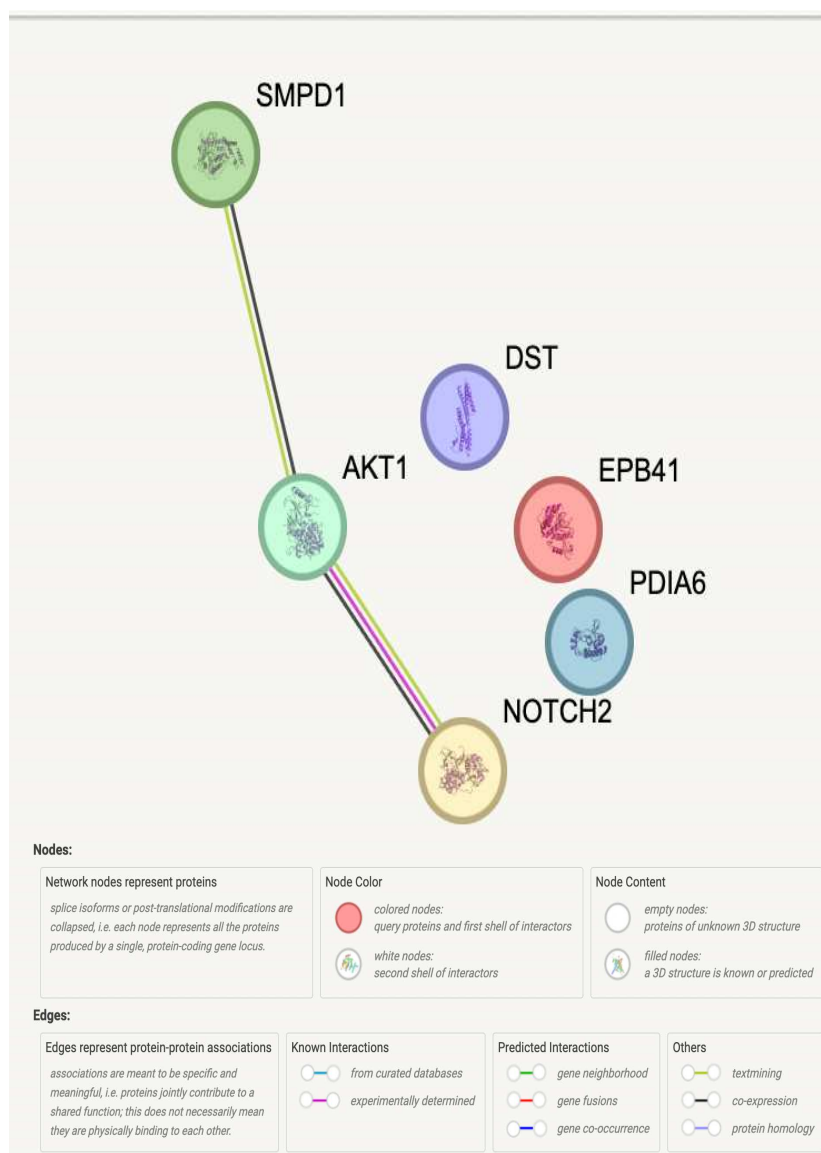


Figure 22: Network of interactions among genes carrying VUS in patient VITT03, along with a corresponding legend

In VITT21 VUS in *COL5A1* and *LRTM2* loci have been observed; interestingly, the *LRTM2* gene encoded the leucine-rich repeat and transmembrane domain-containing protein 2 also involved in heparin binding. Moreover, this patient also displayed a further VUS in the *COL5A1* gene, encoding the alpha 1 chain of the type V collagen, able to bind thrombospondin as well as heparin (Mumby S. M. et al., 1984 – Yao Y. et al., 1990), thus supporting its possible involvement in the clinical phenotype. VITT21 also carries a VUS in the *ADAM8* gene. *ADAM8* gene encodes a member of the *ADAM* (a disintegrin and metalloprotease domain) family, a family of transmembrane glycoproteins with heterogeneous expression profiles and proteolytic, cell-adhesion, -fusion, and -signaling properties. Data from the literature reported that *ADAM8* has immunomodulation properties; in particular, it has been observed to be associated with the function of neutrophils during inflammatory responses (Gómez-Gaviro M. et al., 2007).

In conclusion, WES analysis performed in 6 patients with VITT identifies a number of rare variants which are conditions possibly favoring the prothrombotic state triggered by the exposure to the vaccine.

To the best of our knowledge, no other studies are present in the literature sequencing patients with VITT. Only a recent paper addressed the genetic characteristics of a VITT patient by genotyping more than 870,000 known SNPs in the human genome with an approach not able to identify unknown and rare variants (Mendes-de-Almeida D. P. et al., 2023).

The observation of a burden of VUS for each VITT patient might raise the issue of whether one or more VUS are causative variants, and whether, instead, we should hypothesize a burden of susceptibility genetic factors in the modulation of the clinical phenotype after an acquired condition, such the exposure to adenovirus.

Taken together, these clinical and genetic observations suggest that, although no shared mutational signatures were detected among VITT patients, rare deleterious variants in key pathways related to coagulation, immune activation, and inflammation may modulate individual susceptibility and influence disease severity and clinical outcome.

Data of the present study, to be confirmed and implemented, need functional studies and/or an extension to a larger number of patients, thus allowing a more comprehensive definition of these molecular pathways in modulating clinical manifestations of this condition also in consideration of a possible evaluation of the combined contribution of rare and common variants.

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