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Inhibitor Development in Previously Untreated Patients with Severe Hemophilia A

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Abstract

The thesis sought to gain a deeper understanding of inhibitor development, a major complication of factor VIII (FVIII) replacement therapy in severe hemophilia A. We carried out 3 studies on previously untreated patients (PUPs) with severe hemophilia A, aged under 6 years old and initially inhibitor-free. In chapters I and II, we studied the signatures of IgG subclasses along treatment with FVIII during the first 50 exposure days (EDs), before and after inhibitor development, to identify hallmarks of inhibitor development and inhibitor persistence. Results confirmed IgG4 as the most prominent subclass in patients with inhibitors and suggested that IgG2 is the only subclass that could be considered a hallmark of inhibitor persistence, detectable as early as 2-4 months after inhibitor development and associated with an 11 times higher likelihood of persistence. In chapter III, we carried out an epidemiological cohort study to measure the risk of inhibitor development in patients upon a change of treatment approach consisting in a switch from plasma-derived products (pdFVIII) to recombinant products (rFVIII), promoting the use of each class in its optimal moment. The results showed that a switch after 50 EDs is immunologically safe.

All in all, the evidence obtained along this thesis allowed to make essentially 2 recommendations:

1) To consider IgG2 as a candidate predictor of bad prognosis, associated with inhibitor persistence.

2) To consider a treatment switch in PUPs after 50 EDs from pdFVIII to rFVIII, allowing to combine the features of both product classes, each at their respective beneficial time.

Chapter 0

Introduction to hemophilia

Terminology and biblical texts

Hemophilia A and B are rare inherited bleeding disorders characterized by the deficiency or dysfunction of coagulation factor VIII (FVIII) and factor IX (FIX), respectively. [1-3] According to the activity of the deficient factor, patients are classified into severe, moderate and mild. Severe hemophilias (FVIII and IX coagulant activity <1 IU/dL or below 1% of normal levels) are characterized by spontaneous bleeds, mainly within muscles and joints, resulting in disabling musculoskeletal damage and chronic arthropathy. [3] Moderate (FVIII and IX coagulant activity between 1 and 5 IU/dL) and mild (5-40 IU/dL) hemophilic patients usually experience post-traumatic and post-surgery bleeding events.

Although a modern description of hemophilia appeared only at the beginning of the 19th century, the first records of abnormal bleedings date back to hundreds of years ago. The Talmud (2nd century AD) stated that baby boys could be exempted from circumcision if two of their brothers had previously died from the procedure. [4]

In 1803, John Conrad Otto, an American physician, was the first to report a hemorrhagic bleeding disorder primarily affecting men and running in certain families. In 1813, John Hay, proposed that affected men could pass the trait for a bleeding disorder to their unaffected daughters, having

observed in the pedigree of a man from Massachusetts a sex-linked recessive trend. [5] In 1828 in Switzerland, Friedrich Hopff and his professor Dr. Schonlein, coined the term "hemorrhaphilia" for the condition later shorted to "hemophilia." In 1947, Dr. Alfredo Pavlovsky from Argentina, distinguished the existence of two types of hemophilia - A and B.

A royal disease

Hemophilia is sometimes referred to as "the royal disease," having affected the royal families of England, Germany, Russia and Spain in the 19th and 20th centuries. Queen Victoria of England, who ruled from 1837 to 1901, is believed to have been the carrier of hemophilia B, or FIX deficiency. She passed the trait on to three of her nine children; her affected son died of a hemorrhage after a fall when he was 30, while her carrier daughters Alice and Beatrice passed it on to several of their children. Alice's daughter Alix married Tsar Nicholas of Russia and gave birth to Alexei who had hemophilia. The three were murdered in 1918 during the Bolshevik war along with their four young daughters of the family who had not married. Thus, the European royal line of hemophilia, that passed on through three generations, no longer exists. [6]

Etiology and first treatments

In the early 1900s, people with hemophilia who needed a transfusion received fresh whole blood from a family member, because there was no way to store blood. In those days, life expectancy of these patients was 13 years. Physicians had discovered that patients would respond to infusions of

plasma when given promptly after breakthrough bleeds. Harvard physicians Patek and Taylor discovered the "antihemophilic globulin" in 1937.

By the early 1960s fresh frozen plasma was being transfused to patients. However, each bag of plasma contained so little of the necessary clotting factor that huge volumes had to be administered. Many children would experience severe joint bleeds that were irreversibly crippling and intracranial hemorrhage that was fatal. By 1960, the life expectancy for a person with severe hemophilia was less than 20 years. In 1964, the clotting process was described in detail, introducing the "cascade" and, shortly after, the "waterfall" theories for coagulation. [7,8]

In a breakthrough in 1965, Dr. Judith Graham Pool from Stanford University discovered that the precipitate left from thawing plasma was rich in FVIII. The latter, called "cryoprecipitate" contained a substantial amount of factor in a smaller volume, revolutionizing hemophilia care, making "self-infusion" possible to patients. In 1977, Mannucci and colleagues were the first to use desmopressin for the prevention of bleeding in mild hemophilia A. [9]

The HIV/HCV disaster

By the mid-1980s, approximately half of the people with hemophilia in the US had become infected with human immunodeficiency virus (HIV) and hepatitis C virus (HCV) through infusion of contaminated blood products; thousands died. The disaster of AIDS in the bleeding disorders community did not finish there and lingered through the following decades. Testing for HCV began in 1992 but by then, an estimated 44% of all people with hemophilia had already contracted the virus. Nowadays, with accurate screening methods and purification techniques, the risk of getting

infected with HIV/HCV through factor products is close to zero. More importantly, the discovery and widespread use of antiviral treatments for both viruses allowed the eradication and the optimal long-term control of HCV and HIV, respectively.

The golden era and future perspectives

The treatment of hemophilia saw significant advances from the 1990s onwards. By then, life expectancy for these patients had reached 70 years, almost comparable to non-hemophiliac male peers. Factor products became safer as viral inactivation methods and strict donation screening procedures started to be implemented. In addition, factor products were manufactured using recombinant technologies. These synthetic factors were fully independent from plasma donations. The Food and Drug Administration (FDA) approved the first recombinant FVIII in 1992. By 1995, FVIII prophylaxis treatment, a regimen performed 2-3 times weekly, became more common in fortunate parts of the world, allowing children in developed countries to live with less pain and be spared from the orthopedic damage following chronic joint bleeding. In the last years, new extended half-life products have become available, allowing to pursue therapy once weekly instead of 2-3 times per week.

With the introduction of prophylaxis, the major treatment concern shifted from viral contaminations to the development of alloantibodies against the infused factor, a complication affecting about one-third of previously untreated patient (PUPs) with severe hemophilia A. These antibodies that render treatment ineffective are called inhibitors. More recently, non-replacement therapies have been developed: notably, emicizumab, a bispecific monoclonal antibody mimicking FVIII, that binds

FIXa and FX, has been approved for use in patients both with and without inhibitors, offering a concrete solution for prophylactic treatment in such patients. The only possibility of definitive cure for hemophilia, i.e., gene therapy, has been studied since 2013 and was approved recently for clinical use, although with some safety and efficacy concerns on the long run, notably for hemophilia A.[10-13]

Inhibitors in hemophilia

The long-standing inhibitor complication

Inhibitor development is the natural immune system response to a non-self-protein. The incidence of alloantibodies in the overall population with hemophilia A is approximately 25% to 30%.[14] Patients with severe hemophilia A are more prone to develop inhibitors than moderate and mild patients. PUPs yet to be exposed to FVIII are at greatest risk of inhibitor development within the first 20 exposure days (EDs) to FVIII.[15,16] The immune response to FVIII is polyclonal and consists of the production of immunoglobulins (Ig), notably isotype G (IgG). The evaluation of IgG subclasses alongside FVIII exposure could provide insight into the dynamics of inhibitor development. Notwithstanding the effectiveness of new non-factor replacement therapies in this setting, such strategies only postpone the problem of inhibitor development due to the need of FVIII infusions during breakthrough bleeding events, trauma, or surgery, leaving patients vulnerable during acute events and raising the risk of morbidity and mortality.[17]

Inhibitor development is believed to have multifactorial etiology, explained by a complex interplay between several genetic and environmental risk factors.[18] FVIII genotype, family history of inhibitors, and ethnicity are among the confirmed genetic risk factors playing a significant role in inhibitor development. Null mutations, leading to almost no endogenous FVIII protein production (such as intron 1 and 22 inversions, nonsense mutations, large deletions, or insertions), antecedents of inhibitors in other family members and Black or Latino ethnic background are suggested to predispose to inhibitor development.[19-22] Among the environmental factors, the ones more commonly agreed upon are intensity of treatment at initial exposures, age at first treatment, and "danger signals" (e.g., infection, vaccination, surgery).[23-25] Although the source of FVIII products (i.e., plasma-derived vs recombinant) is still one of the most debated environmental risk factors, the Study on Inhibitors in Plasma-Product Exposed Toddlers (SIPPET) randomized clinical trial provided evidence of a higher immunogenicity rate associated with rFVIII products in PUPs.[26]

Thesis aims

The aim of this three-year PhD journey was to gain a deeper understanding of the inhibitor complication. Firstly, we studied the immunologic response, in particular the behavior of IgG subclasses at different phases of FVIII replacement therapy (chapter I). Secondly, we investigated the IgG subclass profile at the time of inhibitor development and there onward (chapter II). Thirdly, we carried out an epidemiological cohort study to measure the risk of inhibitor development in patients upon a change of treatment approach, since most of the FVIII products used nowadays are recombinant (chapter III). The patients included in all 3 analyses were male PUPs with severe hemophilia A, under 6 years old and inhibitor-free at the beginning of their inclusion in the study.

Chapter I

IgG subclasses as biomarkers for persistence of factor VIII inhibitors in previously untreated patients with severe hemophilia A

Abstract

The immune response to FVIII consists of the development of neutralizing IgG antibodies (IgG1, IgG2, IgG3, and IgG4) in about 30% of PUPs or minimally treated patients (MTPs) with severe hemophilia A, during the first 50 EDs to FVIII replacement therapy. In the 6 months following inhibitor development, one-third of inhibitors disappears spontaneously due to endogenous immune tolerance (transient inhibitors), while two-thirds remain (persistent inhibitors), requiring immune tolerance induction. Although inhibitor persistence impacts therapeutic choices, its mechanisms have been scarcely explored. We investigated the anti-FVIII IgG subclasses longitudinally in the SIPPET cohort in two steps. Step 1 aimed to identify signatures of inhibitor development and hallmarks of persistence. Step 2 aimed to explore how the early/late development of inhibitors and the treatment product impact IgG subclass profiles. Forty patients from the SIPPET cohort were included, of whom 20 had developed inhibitors: 10 transient, 10 persistent. We assessed the IgG subclass profiles along 5 time points: baseline, 5, 10, 20, 50 EDs. IgG4 was the most prominent subclass in inhibitor patients, while IgG2 was the only subclass that distinguished persistent inhibitors. In conclusion, IgG2 showed to be the only subclass that could be considered a promising hallmark of inhibitor persistence, early after inhibitor detection, detectable around ED 20. The confirmation of these results within the SIPPET cohort and other similar cohorts is warranted to validate this observation internally and externally.

Introduction

In severe hemophilia A, the major complication of factor VIII (FVIII) replacement therapy is the development of alloantibodies that neutralize the infused factor and render treatment ineffective.[27] This complication develops predominantly during the first 20 days of exposure to therapy (EDs), in about 30% of previously untreated patients (PUPs) or minimally treated patients (MTPs) with blood components (whole blood, packed red blood cells, platelets, cryoprecipitate or fresh frozen plasma).[28] In one-third of cases, inhibitors are endogenously tolerized and disappear spontaneously within six months (transient inhibitors), while in two-thirds of cases they become persistent. The latter usually require eradication by means of immune tolerance induction (ITI), i.e., frequent infusions of high doses of FVIII to help the immune system recognize the infused factor as self-protein.[29,30] ITI is efficacious in approximately two-thirds of cases but being costly and demanding for both patients and healthcare systems, there is a need to identify predictive factors of inhibitor persistence.[3,31,32]

While several risk factors, some widely accepted[23,33-35] and others under debate[23], have been reported to be associated with inhibitor development, the biology underlying the spontaneous tolerization of some inhibitors and the persisting of others has been scarcely explored. This is partly because most studies investigating the inhibitor complication have focused on patient follow-up until inhibitor development but rarely beyond this time point; if beyond so, they have been often in the context of ITI. To date, across the scanty data, the vast majority has tackled inhibitor development in adults. Studies on inhibitor persistence in PUPs are quite rare. To our knowledge

there is only one international prospective cohort on PUPs, monitoring them until inhibitor development and beyond: The Hemophilia Inhibitor PUPs Study (HIPS).[37]

The immune response to FVIII consists of the production of Ig alloantibodies, that can be neutralizing, leading to the inactivation of the infused factor, or non-neutralizing (i.e., non-inhibitory), targeting non-functional epitopes on FVIII. In humans, of the five Ig isotypes (IgM, IgD, IgG, IgA, and IgE), IgG is the most abundant in human serum, with four subclasses, IgG1, IgG2, IgG3, and IgG4, that have different constant regions. This results in various functions, particularly regarding complement activation and cellular Fc receptor (FcR) binding.[38,39]

Pertaining to immune reactivity towards FVIII in PUPs with severe hemophilia A, the role and behavior of IgG subclasses are yet to be clearly understood, because the available studies yielded poorly comparable results owing to their heterogeneity in design, populations, time points of follow-up, and lack of standardized assays for the detection of subclasses. On the whole, previous findings suggested that anti-FVIII IgG1 and IgG4 are the most prevalent IgG subclasses[40-50] and that IgG2 and IgG3 were less frequently detected in inhibitor patients.[38] Furthermore, high-affinity anti-FVIII specific IgG1 appear first, with a subsequent switch to IgG3 and IgG4.[51] It is poorly known whether the IgG subclass profile alone, could predict inhibitor development and persistence, and how this profile evolves along FVIII exposure in patients with and without inhibitors, of transient or persistent nature. In the current thriving era of novel therapies, the early identification of patients with a higher risk of inhibitor persistence may help to precociously tailor treatment plans, ITI or alternative non-replacement personalized approaches, according to the risk profile of each patient.

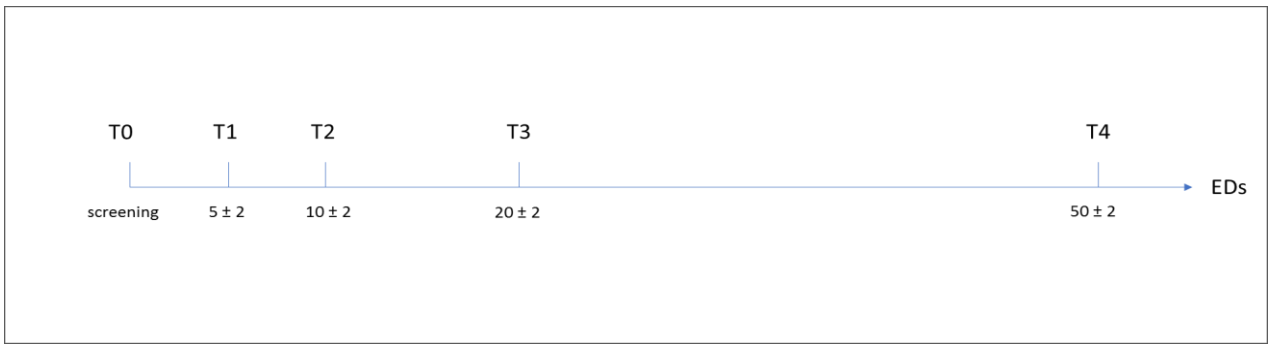
Owing to the SIPPET (Survey of Inhibitors in Plasma-Product Exposed Toddlers) randomized trial[26] and its unique features consisting in the regular collection of plasma samples along FVIII exposure, we chose to investigate the anti-FVIII IgG subclass profile in a subset of patients from this cohort who had sequential samples available at different time points from baseline (before any treatment with FVIII) to 50 EDs or until inhibitor development. This study was designed with two principal aims (Step 1) and three exploratory aims (Step 2). The first aim of Step 1 was to investigate along 5 time points the anti-FVIII IgG subclass signatures in patients who developed inhibitors, while the second aim was to understand whether these IgG subclasses showed specific patterns attributable to patients with persistent inhibitors. In the frame of the exploratory assessment in Step 2, we sought to investigate whether the IgG subclass profiles along the 5 time points differed in patients with low vs high titer inhibitors, early vs late developing inhibitors as well as in patients treated with recombinant (rFVIII) vs plasma-derived (pdFVIII) products.

Materials and Methods

Study design

In this *post-hoc* SIPPET nested case-control study, 40 subjects were included based only on plasma availability at 5 different time points (±2 EDs) as shown in Figure 1: baseline, 5, 10, 20, and 50 EDs. We analysed the IgG subclass profiles at both the individual patient and overall population level. The project received ethical approval from Milan Area 2 Committee.

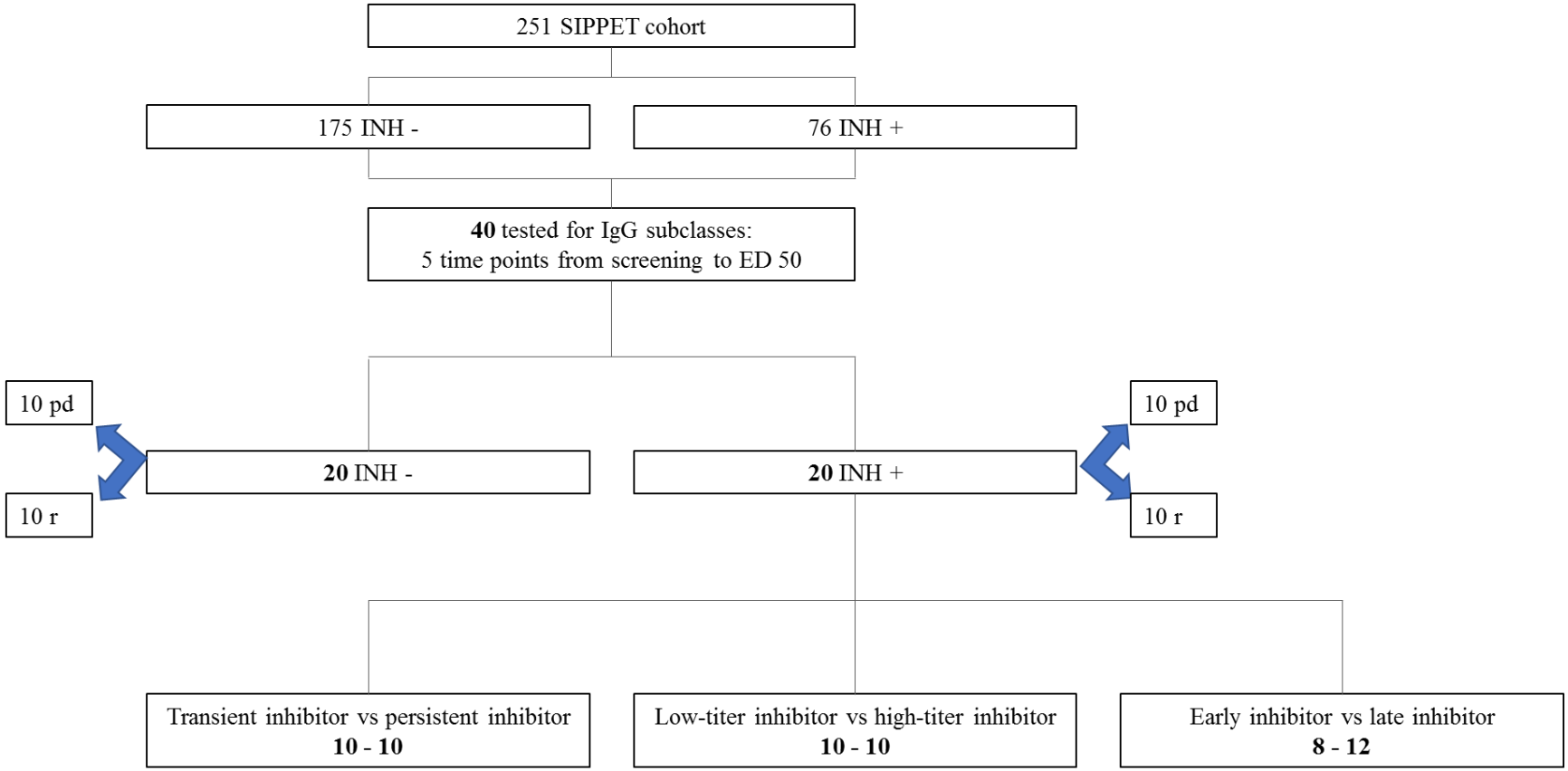
Figure 1. Time points of the longitudinal analysis.



Patients

Patients enrolled in the SIPPET study were male subjects with severe hemophilia A, aged less than six years and were PUPs or MTPs. Patients who underwent randomization were followed for 50 consecutive EDs or 3 years or until inhibitor development as confirmed at the central Milan laboratory, whichever occurred first. An exposure day was defined as a calendar day with one or more infusions of FVIII.[26] The entire SIPPET cohort consisted of 251 patients, of whom 75 developed an inhibitor (Figure 2).

Figure 2. Flowchart of the patients included in Step 1 and Step 2 of the study. INH: inhibitor. Pd: pdFVIII. R: rFVIII.



Baseline characteristics, treatment parameters and main factors known to be associated with inhibitor development are shown in Table 1 for the whole SIPPET population of 251 patients, comprising the 211 patients not included in this nested case-control study due to lack of sequential plasma samples as well as the 40 patients included.

Cases were defined as those patients who developed an inhibitor at any time point during the first 50 EDs, controls were those who remained inhibitor-free. Among the 40 patients included, 20 had developed inhibitors, of whom 10 were transient and 10 persistent.

By SIPPET protocol, patients who developed an inhibitor had to be followed for 6 months during which monthly sample collection was scheduled. In practice, sample collection was suspended after inhibitor development at some centers at the discretion of the local investigator. In the present analysis, this choice hampered the assessment of the last study time point (50 EDs) for 12 out of 20 inhibitor patients. Thus, we chose to focus our observation on the first 20 EDs, time frame in which all patients had available samples. Regarding treatment after inhibitor development, the choice was up to local guidelines and practices outside the study protocol: some continued to be treated with FVIII (none of them underwent ITI), while for others FVIII replacement therapy was suspended.

Table 1. Baseline characteristics, treatment parameters, and factors associated with inhibitor development for different subpopulations of SIPPET, included or not in this analysis. Continuous variables are expressed as medians (IQR). *: The Total EDs of treatment reported here reflects real practice and not total EDs as per protocol. B: Only minor surgeries were reported during the study frame of 50 EDs.

					Step 1				Step 2					
		SIPPET population (n = 251)			Study population (n = 40)		Study population inhibitor + (n = 20)		Study population inhibitor + (n = 20)					
	Characteristics	Inhibitor + (n = 76)	Inhibitor - (n = 175)	All (n = 251)	Inhibitor + (n = 20)	Inhibitor - (n = 20)	Transient (n = 10)	Persistent (n = 10)	Low-titer (n = 10)	High-titer (n = 10)	Early (n = 8)	Late (n = 12)	pdFVIII (n = 10)	rFVIII (n = 10)
Baseline	Type of F8 mutation *N (%)*													
	Null	65 (86)	132 (75)	197 (79)	15 (75)	15 (75)	7 (70)	8 (80)	8 (80)	7 (70)	6 (75)	9 (75)	9 (90)	6 (60)
	Non-null	7 (9)	31 (18)	38 (15)	4 (20)	4 (20)	2 (20)	2 (20)	1 (10)	3 (30)	1 (12.5)	3 (25)	0	4 (40)
	NA	4 (5)	12 (7)	16 (6)	1 (5)	1 (5)	1 (10)	0 (0)	1 (10)	0 (0)	1 (12.5)	0 (0)	1 (10)	0
	Family history of hemophilia *N (%)*													
	No	43 (57)	92 (53)	135 (54)	16 (80)	10 (50)	8 (80)	8 (80)	8 (80)	8 (80)	6 (75)	10 (83)	9 (90)	8 (80)
	Yes	32 (42)	79 (45)	111 (44)	4 (20)	9 (45)	2 (20)	2 (20)	2 (20)	2 (20)	2 (25)	2 (17)	1 (10)	2 (20)
	NA	1 (1)	4 (2)	5 (2)	0	1 (5)	0	0	0	0	0	0	0	0
	Family history of inhibitor *N (%)*													
	No	66 (87)	141 (81)	207 (82)	20 (100)	15 (75)	10 (100)	10 (100)	10 (100)	10 (100)	8 (100)	12 (100)	10 (100)	10 (100)
	Yes	5 (6.5)	20 (11)	25 (10)	0 (0)	3 (15)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0	0
	NA	5 (6.5)	14 (8)	19 (8)	0 (0)	2 (10)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0	0
Treatment	Previous treatment *N (%)*													
	PUP	44 (58)	98 (56)	142 (57)	6 (30)	17 (85)	2 (20)	4 (40)	4 (40)	2 (20)	3 (37.5)	3 (25)	2 (20)	4 (40)
	MTP	32 (42)	77 (44)	109 (43)	14 (70)	3 (15)	8 (80)	6 (60)	6 (60)	8 (80)	5 (62.5)	9 (75)	8 (80)	6 (60)
	Age at first FVIII infusion *(months)*	16 (9–27)	15 (9–29)	15 (9–29)	16 (12–28)	13 (10–31)	14 (10–16)	28 (14–42)	12 (10–15)	28 (20–42)	15 (11–28)	16 (13–26)	20 (13–27)	14 (10–25)
	Total ED*	28 (12–50)	50 (18–50)	22 (8–50)	42 (29–50)	50 (50–50)	43 (38–50)	32 (24–50)	46 (32–50)	39 (28–49)	40 (27–50)	42 (34–50)	39 (30–48)	47 (29–50)
	Type of FVIII product *N (%)*													
	Plasma-derived	29 (38)	96 (55)	125 (50)	10 (50)	10 (50)	4 (40)	6 (60)	4 (40)	6 (60)	5 (62.5)	5 (42)	10 (100)	0
	Recombinant	47 (62)	79 (45)	126 (50)	10 (50)	10 (50)	6 (60)	4 (40)	6 (60)	4 (40)	3 (37.5)	7 (58)	0	10 (100)
	Regimen *N (%)*													
	On-demand	26 (34)	75 (43)	101 (40)	3 (15)	2 (10)	1 (10)	2 (20)	1 (10)	2 (20)	1 (12.5)	2 (17)	1 (10)	2 (20)
	Prophylaxis	50 (66)	100 (57)	150 (60)	17 (85)	18 (90)	9 (90)	8 (80)	9 (90)	8 (80)	7 (87.5)	10 (83)	9 (90)	8 (80)
	Intensive treatment *N (%)*													
	No	71 (93)	166 (95)	237 (94)	19 (95)	19 (95)	10 (100)	9 (90)	10 (100)	9 (90)	8 (100)	11 (92)	10 (100)	9 (90)
	Yes	5 (7)	9 (5)	14 (6)	1 (5)	1 (5)	0	1 (10)	0	1 (10)	0	1 (8)	0	1 (10)
	Surgery *N (%)*													
	No	75 (99)	158 (90)	233 (93)	20 (100)	17 (85)	10 (100)	10 (100)	10 (100)	10 (100)	8 (100)	12 (100)	10 (100)	10 (100)
	Yes[B]	1 (1)	17 (10)	18 (7)	0	3 (15)	0	0	0	0	0	0	0	0
Inhibitor	Age at development *(months)*	20 (14–30)	--	20 (14–30)	20 (16–30)	--	18 (12–20)	30 (20–47)	17 (12–20)	30 (21–47)	19 (12–30)	21 (17–30)	23 (18–29)	19 (12–28)
	ED at development	8 (6–14)	--	8 (6–14)	12 (7–16)	--	12 (10–16)	10 (7–16)	13 (8–16)	11 (7–14)	7 (7–7)	16 (12–17)	9 (7–12)	13 (10–17)
	Titer *peak (Bethesda IU)*	15 (4–87)	--	15 (4–87)	6 (2–19)	--	2 (1–5)	14 (7–39)	2 (1–4)	21 (14–57)	5 (3–30)	8 (2–15)	11 (4–36)	4 (1–12)
	High-titer, *N (%)*	50 (66)	--	50 (66)	10 (50)	--	2 (20)	8 (80)	--	--	4 (50)	6 (50)	4 (40)	6 (60)
	Low-titer, *N (%)*	26 (34)	--	26 (34)	10 (50)	--	8 (80)	2 (20)	--	--	4 (50)	6 (50)	6 (60)	4 (40)
	Persistence *N (%)*													
	Transient	19 (25)	--	19 (25)	10 (50)	--	10 (100)	0	8 (80)	2 (20)	3 (37.5)	7 (58)	4 (40)	6 (60)
	Persistent	52 (68)	--	52 (68)	10 (50)	--	0	10 (100)	2 (20)	8 (80)	5 (62.5)	5 (42)	6 (60)	4 (40)
	NA	5 (7)	--	5 (7)	0	--	0	0	0	0	0	0		
	Development phase *N (%)*													
	Early	49 (64)	--	49 (64)	8 (40)	--	3 (30)	5 (50)	4 (40)	4 (40)	8 (100)	0	3 (30)	5 (50)
	Late	27 (36)	--	27 (36)	12 (60)	--	7 (70)	5 (50)	6 (60)	6 (60)	0	12 (100)	7 (70)	5 (50)

Outcomes: inhibitor development, persistent, high-titer, and late-developing inhibitors

The primary outcome was inhibitor development with a titer ≥ 0.4 Bethesda units by the Bethesda assay with Nijmegen modification.[52] Secondary outcomes were the development of persistent inhibitors, high-titer inhibitors and late-developing inhibitors. High-titer inhibitors were defined as peak levels ≥ 5 Bethesda units during the 6 months following development. Inhibitors most often occur in the first 20 EDs to FVIII; those developing after 50 EDs are considered late-developing[26,53,54]. Since in the overall SIPPET cohort the highest rate of inhibitor development has been reported in the first 10 EDs[26], we defined inhibitors that developed by ED 10 as early-developing and after that as late-developing.

Exposures: product type

In the SIPPET, patients were randomized to treatment with either pdFVIII products containing or not the von Willebrand factor (VWF), chaperone for FVIII stabilization, or with rFVIII products with no VWF. Thus, of the SIPPET participants who completed the study (n=251), half had been assigned to pdFVIII (n=125) and half to rFVIII (n=126).[26]

Laboratory Methods

1. Assay

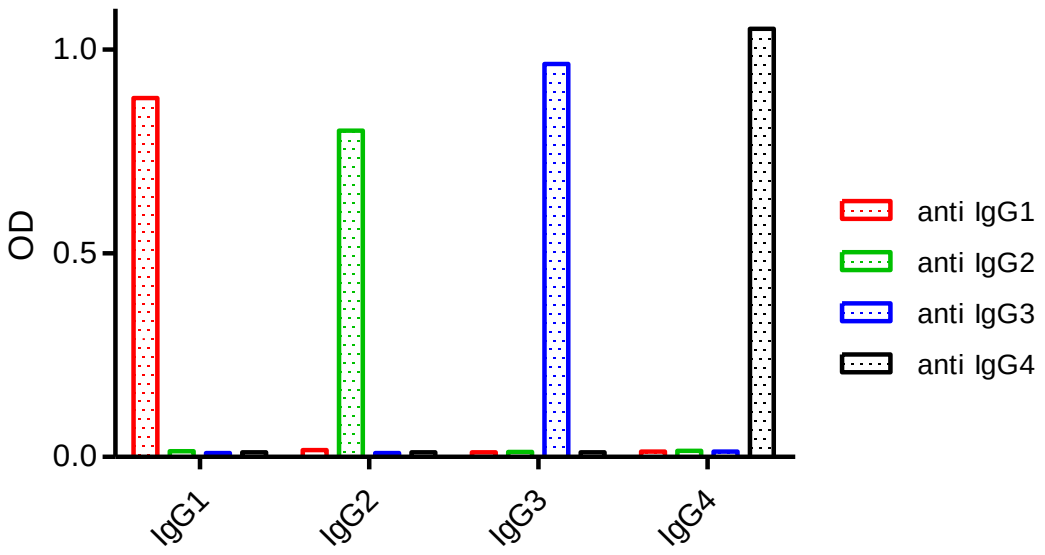
Anti-FVIII IgG subclasses were evaluated by the enzyme linked immunosorbent assay (ELISA) method previously used in the SIPPET context and reported by our group, with minor modifications.[36] Polystyrene plate (MaxiSorp, Nunc, Thermo Fisher Scientific, Roskilde, Denmark)

was coated with the full-length recombinant FVIII product Advate (Baxter Healthcare Corporation, Westlake Village, CA) at the final concentration of 1.2 ug/mL in Phosphate Buffered Saline (PBS) and incubated overnight at 4 °C. After blocking with PBS containing 5% skim milk powder (Merck, Darmstadt, Germany), 2 hours (h) at room temperature (R/T), the plate was washed 5 times with PBS, 0.1% Tween 20 and then, the samples diluted 1/10 in assay buffer (PBS, 1% skim milk powder, 0.01% Tween 20), were loaded into the wells and incubated 2 h at R/T. After washing step, biotinylated mouse anti-human IgG1 (A10650 Life Technology, Carlsbad, California, USA), IgG2 (053540 Invitrogen, Carlsbad, California, USA), IgG3 (053640 Invitrogen, Carlsbad, California, USA), IgG4 (B3648 Sigma and Merck, Kenilworth, NJ) were appropriately diluted, loaded into the wells, and incubated 2 h at R/T. After washing step, high sensitivity Streptavidin-HRP (21130 Pierce, Carlsbad, California, USA) was added and incubated 1 h at R/T. Then, the o-phenylenediamine dihydrochloride substrate (Sigma and Merck, Kenilworth, NJ) was added and after 5 minutes, the reaction was stopped with 3 mol/L H2SO4. Absorbance was read at 492 nm.

2. Specificity of anti-IgG subclass antibodies

All four detection antibodies were tested against four human recombinant IgG subclasses (SIGMA ALDRICH, Missouri, USA) to test their specificity to the appropriate human IgG subclass. There was no cross-reactivity (Figure 3) between the antibodies and the different human recombinant IgG subclasses (1µg/mL).

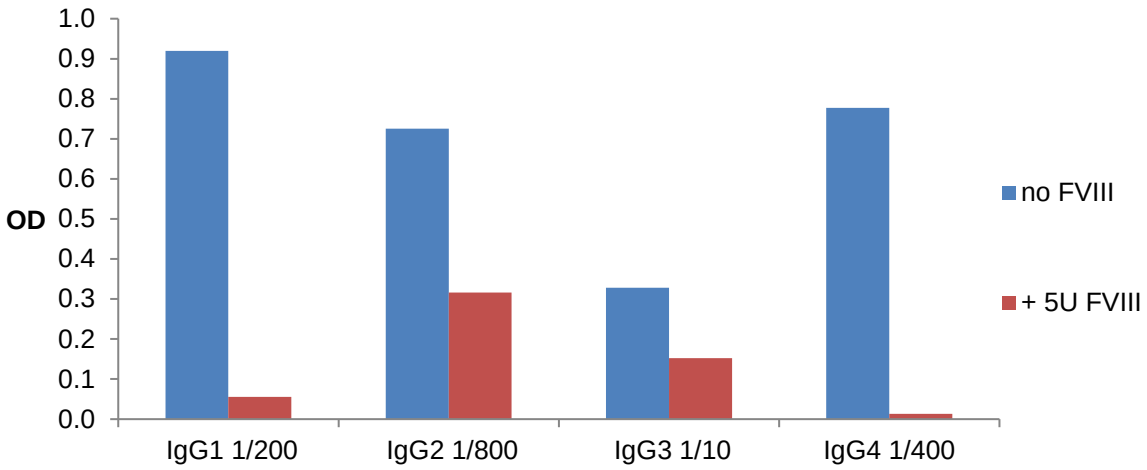
Figure 3. Cross-reaction between the antibodies and the human recombinant IgG subclasses (1µg/mL). OD: optical density.



3. Selection of positive controls

Severe hemophilia A patients, positive for non-neutralizing anti-FVIII IgGs, as detected with the previously reported assay[36], were evaluated for the anti-FVIII IgG subclasses. For each subclass, a sample with absorbance value significantly higher than the cutoff value was identified. The specificity of the signal was confirmed with binding inhibition to the coated FVIII by the mixture of the positive plasma spiked with 5 IU rFVIII (Figure 4). The signal obtained with increasing dilutions of the positive samples (starting dilutions 1/200 for IgG1, 1/800 for IgG2, 1/10 for IgG3 and 1/400 for IgG4) decreased in a dose dependent manner. The positive plasma, included in each assay as positive controls, were measured ten times in ten different plates. The mean value +/- standard deviation (SD) obtained from these measurements established the range of absorbance within which the positive control had to fall to accept and validate the assay.

Figure 4. Determination of cutoff for positivity. Competition was created by the addition of 5 IU of rFVIII before the assay.



4. Selection of negative controls

Among the 150 normal controls used for cutoff determination, four samples were chosen, based on plasma availability, and were included in each assay as negative controls. The four negative plasmas were previously measured ten times in ten different plates. The mean value +/- SD obtained from these measurements established the range of absorbance within which the negative control had to fall to accept and validate the assay.

5. Determination of cutoff for positivity

A cutoff for positivity was calculated, for each anti-FVIII IgG subclass, based on the absorbance levels obtained in 150 healthy subjects. The initial cutoff, determined as the mean + 3 SD of the absorbance values of the 150 normal controls, was very close to the detection limit of the ELISA reader, mostly for IgG4. Hence, the new cutoff was established as the mean + 5 SD for all

subclasses. This allowed to increase the robustness of the assays maintaining similar sensitivity (only one normal sample gave an absorbance value slightly above the new cutoff).

6. Daily quality controls

In each assay one positive and four negative controls were included. The assay was validated if their absorbance values were within the range of values established as described above (points 3 and 4).

7. Precision of ELISA tests

The inter-assay variation was calculated by means of 26 independent assays resulting in a coefficient of variation between 9.2 to 13% for the positive control and from 12 to 24.5% for the negative control among the four IgG subclasses. The intra-assay variation, obtained by testing ten times in the same plate both the positive controls and the four negative controls, resulted in a coefficient of variation spanning from 2.2 to 2.7% for the positive control and from 5.4 to 9.2% for the negative control (Table 2). In order to measure the relative contribution of each subclass without the influence of the inter-assay variability, each patient was tested for the four anti-FVIII IgG subclasses in the same plate.

Table 2. Precision of the ELISA tests. The coefficients of variation (%) are indicated. *mean of the four normal controls included in each assay.

	inter-assays		intra-assay	
IgG subclass	**positive control**	**negative control***	**positive control**	**negative control***
IgG1	10.9	12.0	2.2	9.2
IgG2	9.2	24.5	2.6	6.3
IgG3	13.0	18.4	2.7	5.4
IgG4	10.8	15.5	2.2	9.2

Statistical Analysis

1. Baseline characteristics

Being this a nested case-control study on 40 patients included based on plasma sample availability, with 20 inhibitor patients and 20 inhibitor-free patients as controls, the distribution of baseline characteristics, treatment parameters and main factors associated with inhibitor development were compared in our study population (n=20 inhibitor patients) with the population of inhibitor cases in SIPPET (n=76 inhibitor patients). The same comparison using Student's *t-test* for medians and chi-square for distribution of proportions was done for controls: our study controls (n=20 inhibitor-free patients) and the whole SIPPET population (n=251).

Descriptive statistics were reported as mean/standard deviation, median/interquartile range (IQR), or as proportions. Baseline characteristics considered and compared were the following variables: type of *F8* gene mutation (null allele comprised inversions, large deletions, frameshifts and

nonsense mutations), family history of hemophilia and of the inhibitor. Treatment characteristics were the following variables: none or previous minimal treatment with blood components (PUP vs MTP), age at first FVIII infusion, total EDs of treatment during the observation period, type of FVIII product (pdFVIII vs rFVIII), regimen (on-demand after breakthrough bleed vs prophylaxis), intensive treatment (defined as ≥ 5 consecutive daily doses of ≥ 50 IU/kg) and surgery (minor or major). Variables regarding inhibitor development were defined as follows: age at inhibitor development, ED at inhibitor development, peak titer (high-titer vs low-titer), inhibitor persistence (transient vs persistent), phase of inhibitor development (early (by ED 10) vs late (after ED 10)).

2. Survival analysis

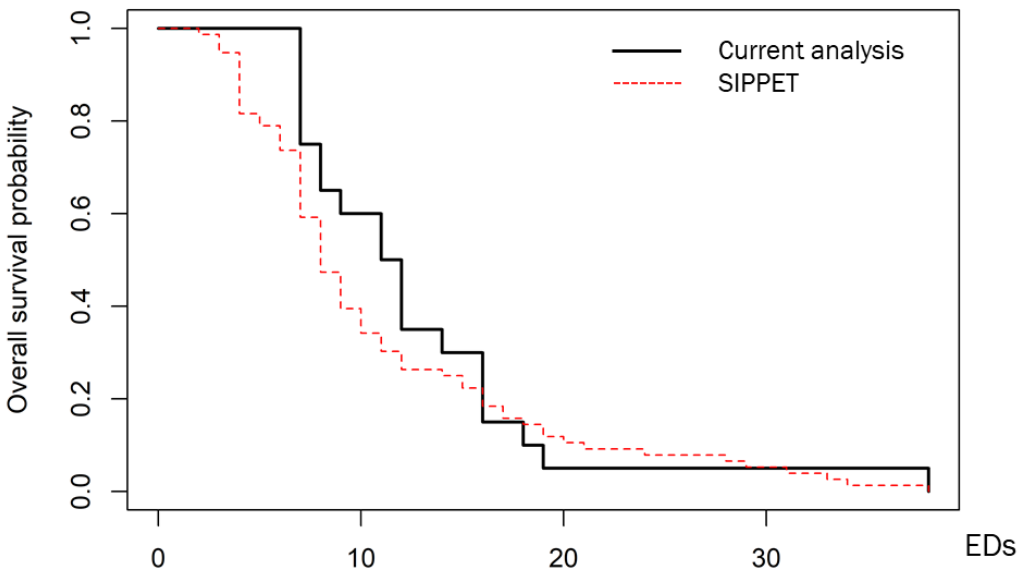
To compare if the outcome-free survival curve of the 20 inhibitor patients in this study was similar to that of the 76 inhibitor patients in the SIPPET cohort, Kaplan-Meier curves were plotted for both groups. Differences between groups with regards to the proportion of patients who were positive for a given IgG subclass at each time point were shown visually using bar graphs at the individual patient and overall population levels. To test if the difference between two groups at a given time point was statistically significant, a chi-square test was performed.

Results

Study population

Comparisons between the characteristics of the cases in our study population (n=20) with the whole SIPPET population of inhibitor patients (n=76), as well as the comparison of our controls (n=20) with the whole SIPPET (n=251) (Table 1) showed no clinically relevant differences. The variables that showed some degree of heterogeneous distribution were previous treatments (PUP vs MTP), ED at inhibitor development and inhibitor peak titer. Survival analysis showing the ED of inhibitor development in our inhibitor population and that of the whole SIPPET inhibitor population is depicted in Figure 5, showing similar tendencies notably the development of almost all inhibitors within 20 EDs. In the study population, no patient developed an inhibitor before ED 5, 40% (8/20) developed an inhibitor between 5 and 10 EDs, and the remaining 55% (11/20) developed an inhibitor between 10 and 20 EDs. Only one patient developed a late inhibitor at ED 38.

Figure 5. Survival analysis in our SIPPET subpopulation under study who developed inhibitors and of the entire SIPPET population.



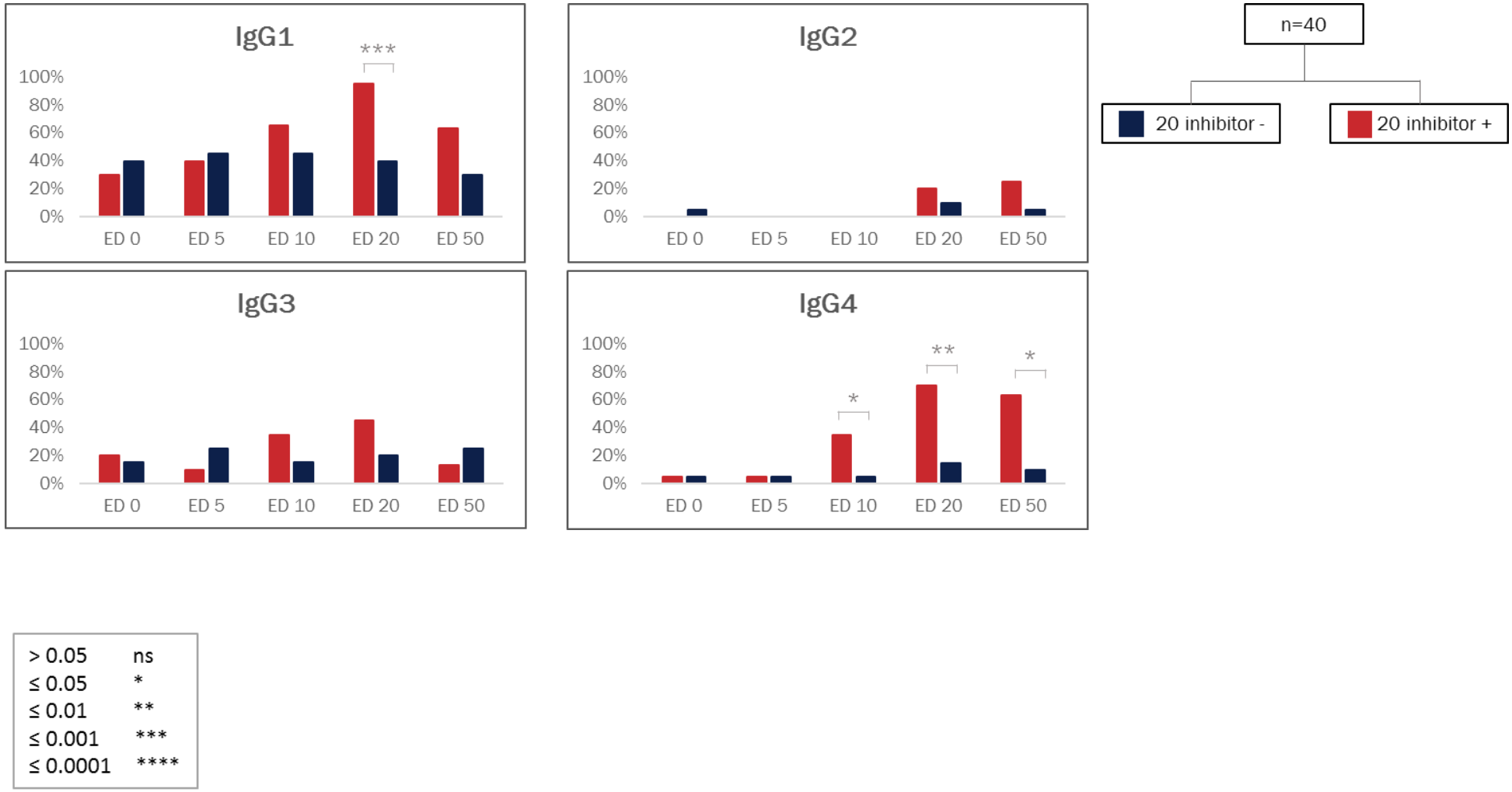
Step 1

I. Inhibitor vs no inhibitor

In the study population of 40 patients, the IgG subclass profile of the 20 cases who had developed inhibitors were compared with the IgG subclass profiles of those inhibitor-free. Figure 6 shows the IgG subclass profile based on presence/absence of inhibitor and demonstrates that IgG1 and IgG3 were detectable in a very small number of inhibitor-free patients and that their prevalence was relatively constant along the 5 time points. IgG1 and IgG4 were the most common subclasses in inhibitor patients, inhibitor development being mainly characterized by the presence of the IgG4 subclass. IgG2 was the most seldom detected subclass and when detected it was usually in a small number of patients.

Individual IgG subclass profiles for all the 20 patients who developed inhibitors are shown in supplementary Figure 7. IgG1 and IgG3 were the only subclasses detectable before inhibitor development. IgG3 was usually detectable after IgG1, IgG4 after IgG1 at the same time as IgG3 or after it, whereas IgG2 was detectable after IgG1 at same time as IgG3 and IgG4 or after IgG3 and IgG4.

Figure 6. IgG subclass profiles in inhibitor patients (n=20) and inhibitor free patients (n=20) from the SIPPET.



II. Transient vs persistent inhibitors

Figure 8 shows the IgG subclass profile based on inhibitor transience/persistence and shows that IgG2 is the only subclass able to differentiate these two groups.

IgG2 was detectable after inhibitor development (ED 20 onwards) only in patients with persistent inhibitors, except for a patient with a transient inhibitor who had detectable IgG2 at ED 20 but then lost it by ED 50. In 90% of patients with persistent inhibitors, individual subclass profiles revealed the presence of at least 3 different subclasses in the time span of 50 EDs, suggesting that a higher combination of different IgG subclasses could be associated with inhibitor persistence. The different scenarios of sequential subclass detection as observed in the transient and persistent inhibitors are shown in Table 3.

Figure 8. IgG subclass profiles in persistent inhibitors (n=10) and transient inhibitors (n=10) from the SIPPET.

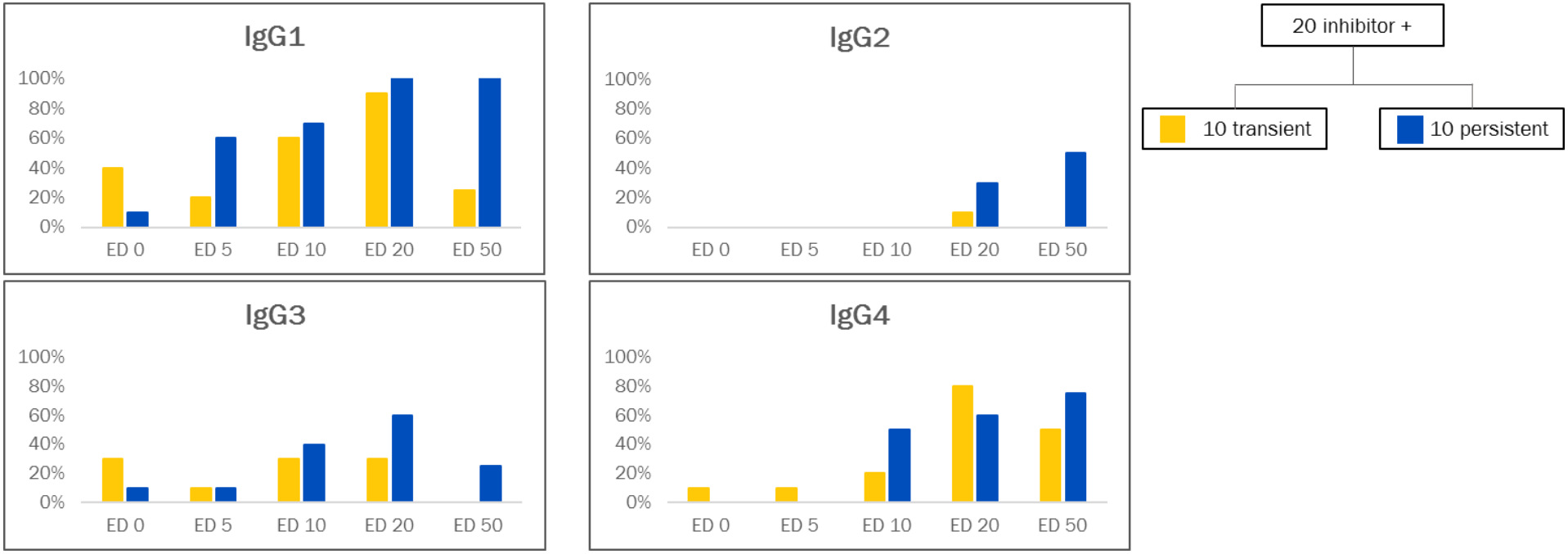


Table 3. Different scenarios of sequential subclass detection observed in the transient and persistent inhibitors. Red arrow: inhibitor development. N: number of patients in whom the respective switch scenario was observed.

Inhibitor patients (n=20)	Transient (n=10)	Persistent (n=10)
Switch scenarios	n	n
IgG1 + IgG3 → ↓ → IgG4 ↓ → *IgG1 + IgG3 → IgG4*	2	3
IgG1 → ↓ IgG3 + IgG4 *IgG1 → ↓ IgG3 → IgG4*	1	2
IgG1 → ↓ → IgG2 + IgG3 ↓ → *IgG1 + IgG2 + IgG3*	0	2
IgG1 → ↓ → IgG4 → IgG2	0	1
IgG1 → ↓ → IgG2 + IgG3 + IgG4	1	1
↓ → IgG1 + IgG4	2	1
↓ → IgG1	2	0
IgG3 → ↓ → IgG4	1	0
IgG1 + IgG3 + IgG4 → ↓	1	0
At least 3 subclasses	5	9

Step 2

I. <u>Low vs high titer inhibitors</u>

Figure 9 shows the IgG subclass profile based on low/high inhibitor titer. The different scenarios of sequential subclass detection as observed in the low and high titer inhibitors are shown in Table 4. We assessed whether they correlated with the profiles based on inhibitor persistence: the profiles based on low/high inhibitor titer were indeed very similar to those based on transience/persistence.

Figure 9. IgG subclass profiles in low-titer inhibitors (n=10) and high-titer inhibitors (n=10) from the SIPPET

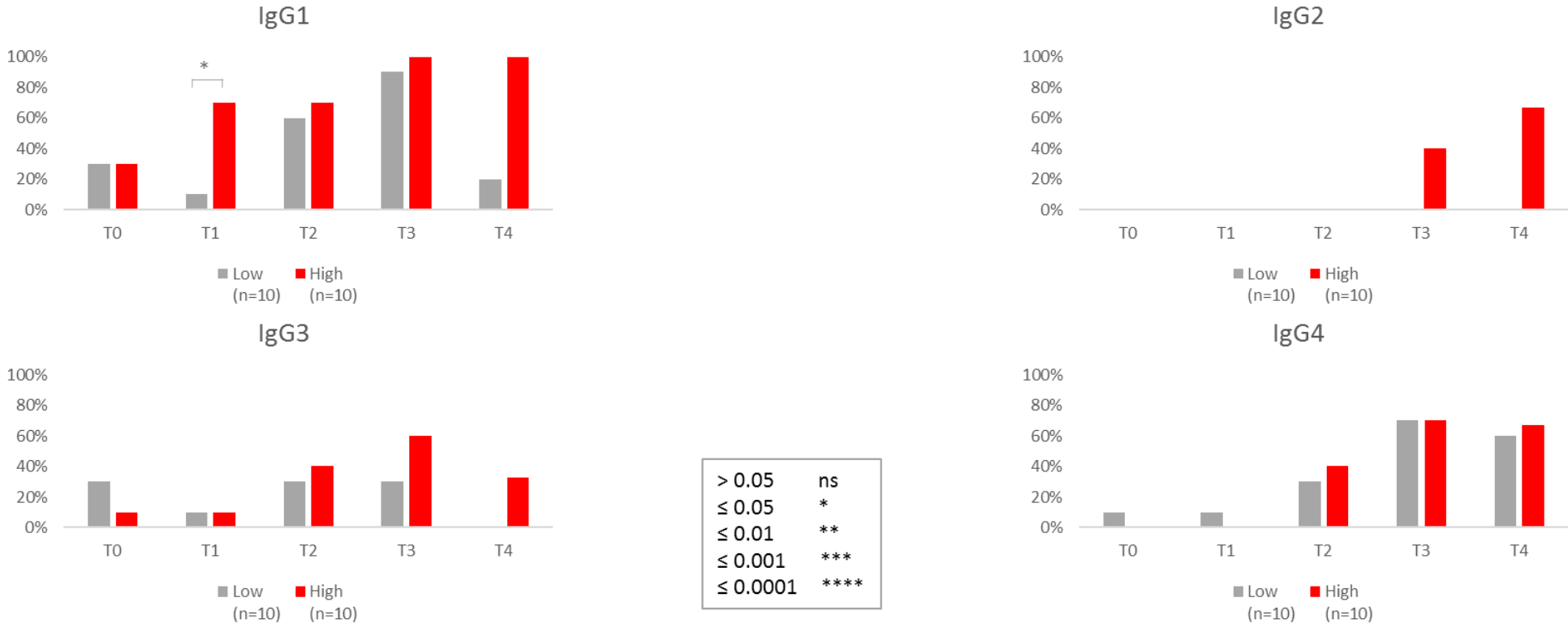


Table 4. Different scenarios of sequential subclass detection observed in the low-titer and high-titer inhibitors. Red arrow: inhibitor development. N: number of patients in whom the respective switch scenario was observed.

Inhibitor patients (n=20)	Low-titer (n=10)	High-titer (n=10)
Switch scenarios	n	n
IgG1 + IgG3 → ↓ → IgG4 ↓ → *IgG1 + IgG3 → IgG4*	3	2
IgG1 → ↓ IgG3 + IgG4 *IgG1 → ↓ IgG3 → IgG4*	1	2
IgG1 → ↓ → IgG2 + IgG3 ↓ → *IgG1 + IgG2 + IgG3*	0	2
IgG1 → ↓ → IgG4 → IgG2	0	1
IgG1 → ↓ → IgG2 + IgG3 + IgG4	0	2
↓ → IgG1 + IgG4	2	1
↓ → IgG1	2	0
IgG3 → ↓ → IgG4	1	0
IgG1 + IgG3 + IgG4 → ↓	1	0
At least 3 subclasses	5	9

II. Early vs late inhibitors

Figure 10 shows the IgG subclass profile based on the early vs late development of the inhibitor. The different scenarios of sequential subclass detection as observed in the early and late developing inhibitors are shown in Table 5. A similar trend was seen in the early and late inhibitor groups for all 4 subclasses. The only apparent difference was a delay in the production of anti-FVIII antibodies rather than a true difference in the subclass composition. The prevalence of IgG1 and IgG3 increased in both the early and late inhibitors until inhibitor development. Afterwards these subclasses became less prominent, while IgG2 and IgG4 were detectable only post inhibitor development, with a prevalence that increased over time in this phase.

Figure 10. IgG subclass profiles in early-developing inhibitors (n=8) and late-developing inhibitors (n=12) from the SIPPET. Red dashed line: time point by which all early-developing inhibitors had emerged. Grey dashed line: time point by which 11/12 (92%) of late-developing inhibitors had emerged (1 inhibitor at ED 38).

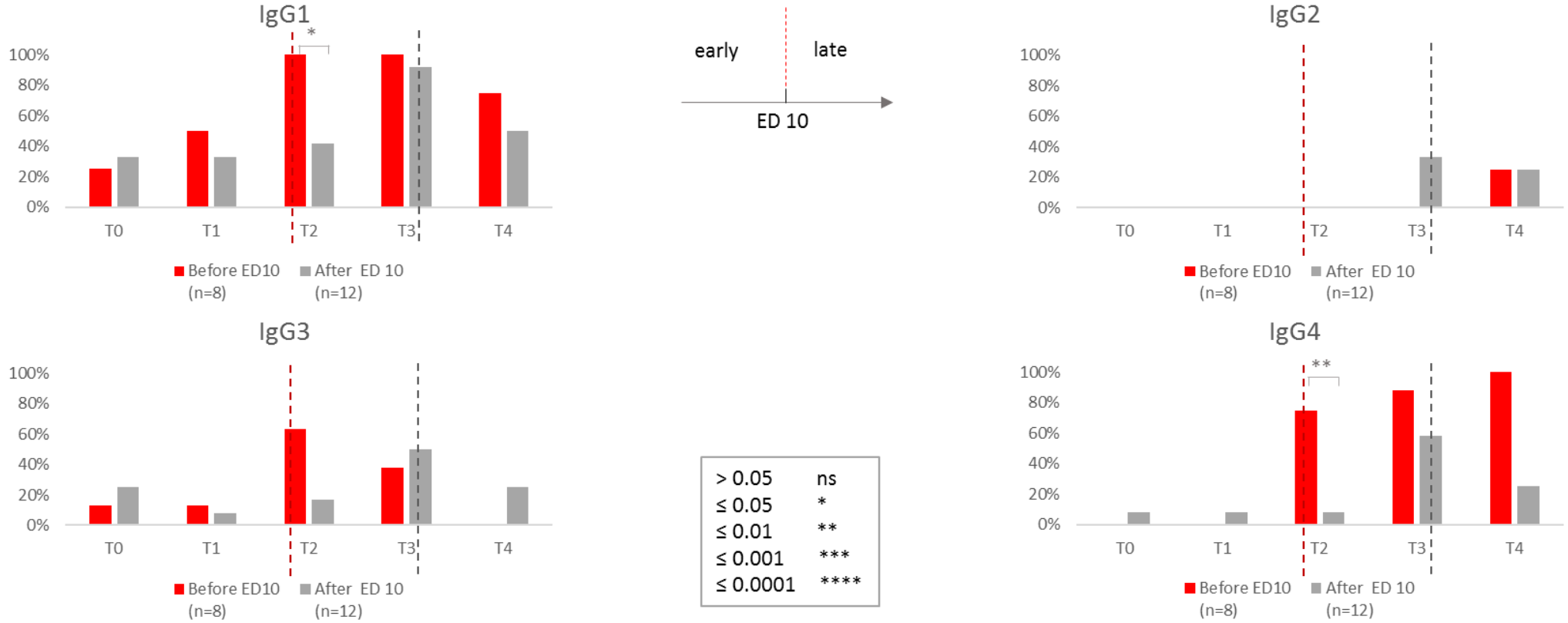


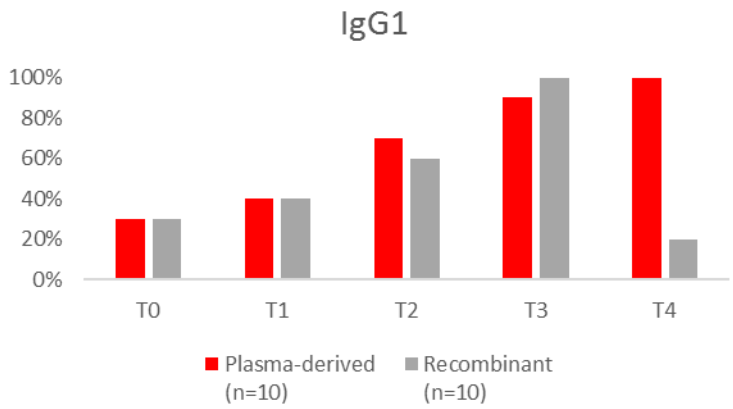
Table 5. Different scenarios of sequential subclass detection observed in the early-developing and late-developing inhibitors. Red arrow: inhibitor development. N: number of patients in whom the respective switch scenario was observed.

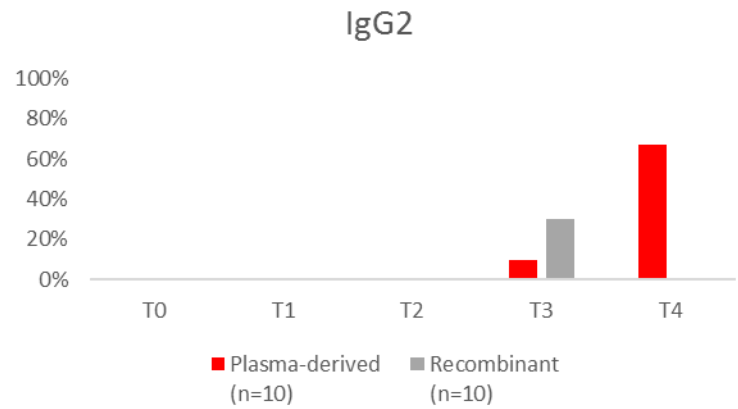
Inhibitor patients (n=20)	Early (n=8)	Late (n=12)
Switch scenarios	n	n
IgG1 + IgG3 → ↓ → IgG4 ↓ → *IgG1 + IgG3 → IgG4*	2	3
IgG1 → ↓ IgG3 + IgG4 *IgG1 → ↓ IgG3 → IgG4*	3	0
IgG1 → ↓ → IgG2 + IgG3 ↓ → *IgG1 + IgG2 + IgG3*	0	2
IgG1 → ↓ → IgG4 → IgG2	1	0
IgG1 → ↓ → IgG2 + IgG3 + IgG4	0	2
↓ → IgG1 + IgG4	2	1
↓ → IgG1	0	2
IgG3 → ↓ → IgG4	0	1
IgG1 + IgG3 + IgG4 → ↓	0	1

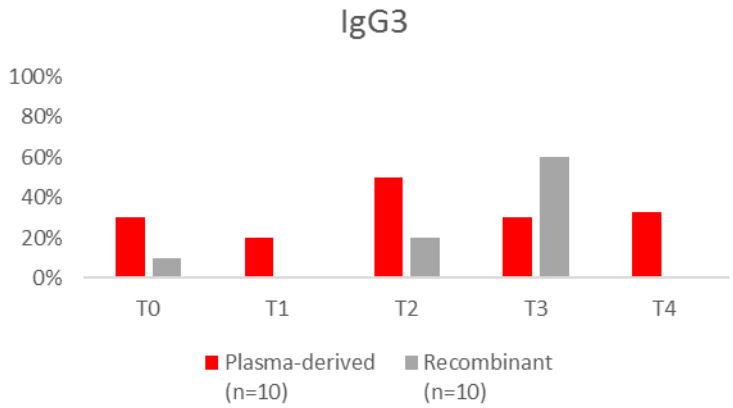
III. Treatment with pdFVIII vs rFVIII

Figure 11 shows the IgG subclass profile in patients treated with pdFVIII vs rFVIII. The different scenarios of sequential subclass detection as observed in these two groups are shown in Table 6 and indicate that the use of both FVIII product classes led to very similar signatures of the IgG subclasses, suggesting that according to this analysis the higher immunogenicity of rFVIII reported in the SIPPET cannot be explained by a difference in the anti-FVIII subclass profile.

Figure 11. IgG subclass profiles in inhibitor patients treated with pdFVIII (n=10) and rFVIII (n=10) from the SIPPET.







> 0.05	ns
≤ 0.05	*
≤ 0.01	**
≤ 0.001	***
≤ 0.0001	****

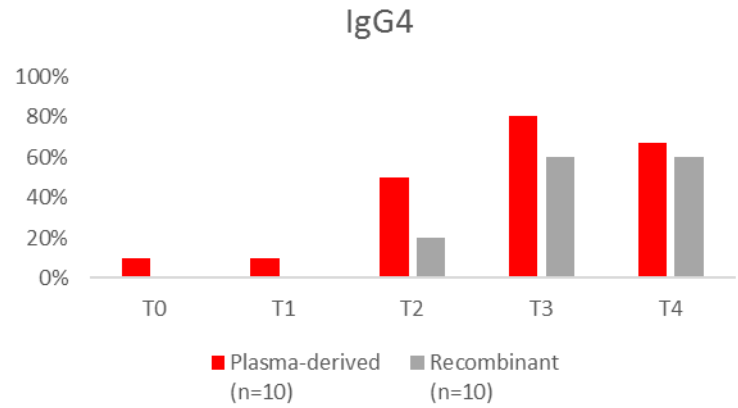


Table 6. Different scenarios of sequential subclass detection observed in patients with inhibitors treated with pdFVIII or rFVIII. Red arrow: inhibitor development. N: number of patients in whom the respective switch scenario was observed.

Inhibitor patients (n=20)	pdFVIII (n=10)	rFVIII (n=10)
Switch scenarios	n	n
IgG1 + IgG3 → ↓ → IgG4 ↓ → *IgG1 + IgG3 → IgG4*	2	3
IgG1 → ↓ IgG3 + IgG4 *IgG1 → ↓ IgG3 → IgG4*	2	1
IgG1 → ↓ → IgG2 + IgG3 ↓ → *IgG1 + IgG2 + IgG3*	1	1
IgG1 → ↓ → IgG4 → IgG2	1	0
IgG1 → ↓ → IgG2 + IgG3 + IgG4	0	2
↓ → IgG1 + IgG4	2	1
↓ → IgG1	0	2
IgG3 → ↓ → IgG4	1	0
IgG1 + IgG3 + IgG4 → ↓	1	0

Discussion

In this *post-hoc* multiple time point analysis on the IgG subclasses in the SIPPET context, we report IgG4 as the most prominent subclass in patients who developed inhibitors, while IgG2 is the only subclass that distinguishes those at risk of developing persistent inhibitors. Furthermore, we report that the difference observed in the IgG subclass profile of early and late inhibitor patients featured a delay in the production of alloantibodies rather than a difference in subclass composition, and that the use of both pdFVIII and rFVIII products led to similar signatures of the IgG subclasses.

Inhibitor development remains the main complication in the treatment of hemophilia. Several studies including SIPPET helped provide a better understanding of the features of inhibitor development. Inhibitors usually occur in young children during the first 20 EDs to FVIII replacement therapy[53,54,56,57], but in the SIPPET population this window narrowed down to the first 20 EDs[26]. Two-thirds of anti-FVIII inhibitors become persistent and usually require ITI therapy but approximately one-third is transient and disappears spontaneously. Because of the paucity of longitudinal studies on inhibitor development, the multi-causal phenomenon of persistence remains unclear. So far, the most cogent finding was that a high titer of anti-FVIII antibodies at inhibitor detection is associated with a higher likelihood of persistence.[16,30,36,44,58,59]

Our identification of IgG4 as the prominent subclass in patients with inhibitors is consistent with the reports in the literature on cohorts of hemophilia A patients with inhibitors.[40-50]

Our findings are in accordance with the only similar study, the HIPS.[51] HIPS is a prospective cohort study assessing profiles of the genome, transcriptome, and comprehensive changes in the immune system of 23 PUPs followed from baseline until 50 EDs at specific time points of exposure (0, 1, 5,

10, 20, 30, 40, 50 EDs) to a single source of full-length standard half-life rFVIII product.[51] In the two transient inhibitor patients reported in HIPS, no switch from IgG1 to other subclasses was reported.[60] However, the small number of patients with transient inhibitors in HIPS (only 2) may explain why a switch from IgG1 to other subclasses was not detected. Regarding the time sequence of switching in the persistent inhibitor group, HIPS and our study both support the initial appearance of IgG1 followed by IgG3 and IgG4 and in some cases IgG2 after IgG1 or after IgG3 and IG4. Similar behavior for IgG2 and IgG4 have been reported in the context of other autoimmune disorders, notably in multiple sclerosis, where the involvement of these two subclasses in a persistent neutralizing immune response to treatment products is suggested. In patients with multiple sclerosis, treatment with recombinant interferonβ 1b induces the production of neutralizing and non-neutralizing anti-drug antibodies. In 2001, an Austrian research group reported a strong correlation between IgG4 and a high titer of neutralizing antibodies.[58] Subsequently, they also reported that patients with transient neutralizing antibodies displayed predominantly IgG1 and IgG3 subclasses and lower titers, compared to patients with persistent neutralizing antibodies in whom the most frequent IgG subclasses were IgG2 and IgG4.[59]

Despite significant insights into the details of humoral immunity, no proposal has emerged that clearly describes how IgG antibody subclasses and other antibody isotypes work together to provide immunocompetence.[39] So far, findings support the notion that IgG1 and IgG3 are potent immune-activating subclasses because they trigger complement activation and Fc receptor binding, while IgG2 and IgG4 have instead regulatory functions in establishing an efficient generation of memory response.[61,62] A temporal model of human IgG function by Collins and colleagues proposed that

after emergence of IgG3, IgG1 cells switch and emerge, followed by IgG2-committed cells (that slow the inflammatory drive) and finally, if the antigen persists, by IgG4-producing cells.[39] For a comprehensive understanding of the mechanisms of inhibitor persistence, the hypothesis of a functional role for IgG2 alone is an underestimation of the complexity of this multifactorial phenomenon. The presence of IgG2 in inhibitors that became persistent may be a by-product of other components such as the Toll-like receptors and cytokines, that in turn may be at the origin of the trigger skewing towards a switch to this subclass in a signaling environment that favors long-lasting response to FVIII. All in all, these findings stimulate an investigation into understanding how these upstream components work together in selectively triggering a switch to IgG2 in patients whose inhibitors are becoming persistent. In the meantime, our results could help to stratify patients with a higher risk of inhibitor persistence.

Limitations of this study are its small sample size and the incomplete assessment of the last study time point due to discontinuation of FVIII exposure in some patients prior to 50 EDs. This notwithstanding, its strength is that so far this is the largest longitudinal multiple time point study designed to characterize inhibitor persistence by comparing a transient inhibitor population with a persistent population in PUPs with severe hemophilia A.

Conclusion

Results from this *post-hoc* SIPPET analysis suggest for the first time that IgG2 is the only subclass that could be considered a hallmark of inhibitor persistence, early after inhibitor detection. The assessment of this hallmark in persistent inhibitors of similar cohorts is warranted in the future to externally validate this observation.

With these results suggesting anti-FVIII IgG subclasses as potential biomarkers for inhibitor development and persistence, we decided to focus on the post inhibitor development phase in a different subset of patients from the SIPPET cohort to see if an internal validation of our previous results could be obtained and furthermore to focus specifically the second analysis on the 6 months following inhibitor development which is the window defining the transient/persistent nature of the inhibitor. This time, instead of defining the time lapse as exposure days, we set it as days from inhibitor development, focusing exclusively on inhibitor patients (chapter II) in the six months following detection, unlike the first study (chapter I) where we started the analysis at baseline and included patients both with and without the inhibitor.

Chapter II

IgG2 as early hallmark of inhibitor persistence in previously untreated patients with severe hemophilia A

Abstract

When exposed to factor VIII (FVIII) products, more than 30% of previously untreated patients (PUPs) with severe hemophilia A develop alloantibodies that neutralize FVIII coagulant activity (inhibitors). Two-thirds of these become persistent whereas one-third is transient. We investigated the IgG subclass profile longitudinally within six months from inhibitor detection in a subset of patients from the SIPPET, different from those included in the first study (chapter I). We first analyzed samples obtained within 60 days following inhibitor detection from 43 patients (12 with transient and 31 with persistent inhibitors). Subsequently, 14 of these 43 patients (five with transient and nine with persistent inhibitors) were studied at five time points over the six months after inhibitor detection. During the first 60 days, the risk of inhibitor persistence increased in parallel with the number of IgG subclasses. Over the subsequent period of six months, the IgG subclass profile showed a predominance of IgG1 and IgG4 in all inhibitor patients, regardless of transience/persistence; IgG3 progressively decreased in both groups. IgG2 was the only subclass that had a differential pattern, in that it disappeared in transient but increased in persistent inhibitors. In conclusion, within 60 days from inhibitor formation, the presence of more than one anti-FVIII IgG subclass was associated with an increased risk of inhibitor persistence. Over the period of six months post inhibitor detection, IgG2 was the only IgG subclass that could be considered a hallmark of inhibitor persistence.

Introduction

Persistent inhibitors represent a true challenge for the clinician who, without having at disposal a crystal ball allowing to see through the future, needs to tailor therapy modalities, non-cognizant of the transient or persistent nature of the inhibitor. The difference of initiating ITI or not lies in stepping or not into a burdensome infusion journey that would consist in daily intravenous administration of high-dose FVIII. This would impinge the patients' quality of life, with success rates varying between 60 – 80%, not to mention the costliness of this approach to the national health system, which translates into its costliness for fellow taxpayers.[63]

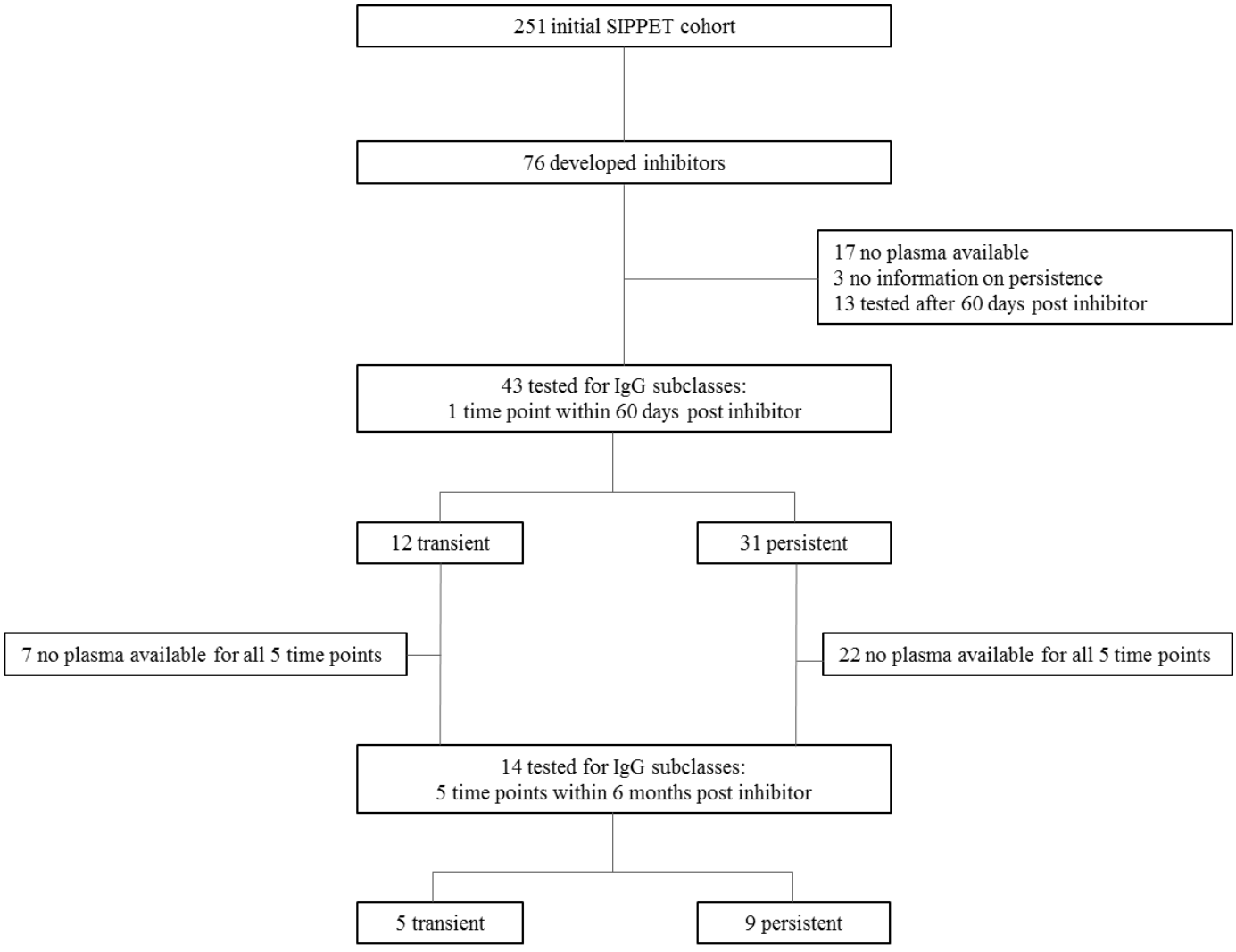
With chapter I results in mind, we decided to study the dynamics of the anti-FVIII IgG subclass profiles, zooming into the inhibitor development moment and the following 6 months. With the unique advantage of sequential plasma sample collection in the SIPPET and with some luck to still have availability of plasma samples from some participants, we designed a second longitudinal analysis to study a subset of patients different from the ones studied in chapter I. The advantage was the possibility to have an internal validation of chapter I results, upon confirmation of the previous observations.

Materials and Methods

Study flowchart

The study flowchart is shown in Figure 1.

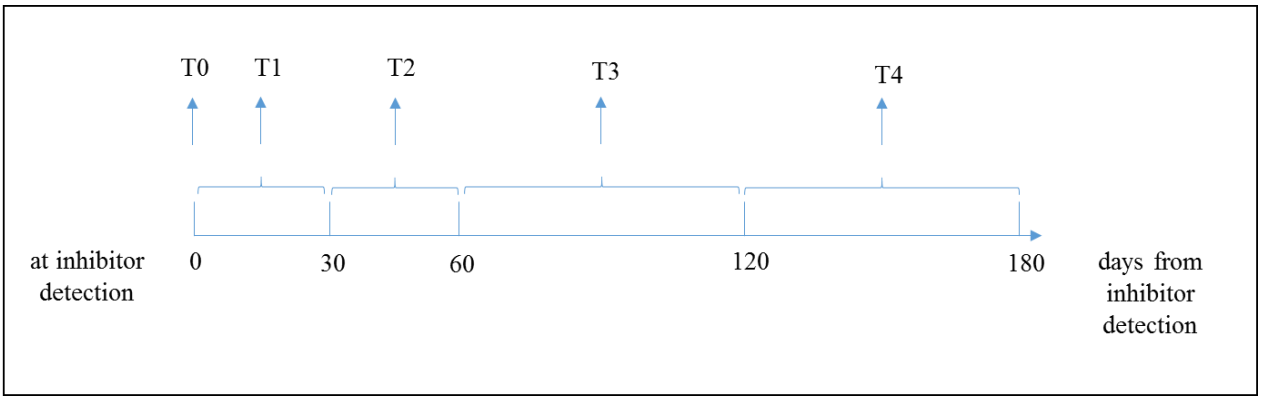
Figure 1. Flowchart.



We first investigated the value of anti-FVIII IgG subclass composition, alone or in combination with other putative factors, on the persistence of FVIII inhibitors in the SIPPET cohort.[18,24,34] This initial study, herein referred to as Step 1, was carried out on one sample obtained within 60 days post inhibitor detection. Subsequently, this was expanded to a sequential analysis of five time points

up to six months after inhibitor detection, herein referred to as Step 2. The first time point of Step 2 was at first inhibitor detection, followed by two time points within 60 days (with average intervals of one month between them), and two additional time points between 60 and 180 days (with an average two-month time interval between them) (Figure 2). The project received ethical approval from Milan Area 2 Committee.

Figure 2. Schematic representation of the five time points assessed in Step 2. When more than one sample in the considered time interval was available, the sample closest to the mean time point for that interval (15, 46, 86, 158 days) was chosen for the analysis.



Step 1

Based only on residual plasma availability, 43 of the 76 patients who developed an inhibitor were analyzed. Among them, 31 had persistent and 12 had transient inhibitors. Baseline characteristics of these 43 patients are provided in Table 1, including the main putative factors associated with inhibitor development. These characteristics showed no significant imbalance between the 43 subjects selected for Step 1 and the rest of the inhibitor patients not included because of sample shortage. A single sample obtained within 60 days [median 19 days (interquartile range 13–36)]

from first inhibitor detection was analyzed to evaluate the association between IgG subclass combination and inhibitor persistence.

Step 2

Additional samples over time from inhibitor detection up to 180 days after were assessed to study anti-FVIII IgG subclass profile evolution and to explore the potential predictive role of each subclass in the frame of persistent/transient anti-FVIII immunogenicity. This analysis was performed in only 14 of the 43 patients from Step 1, due to limited sample availability. Among the 14 patients, nine had persistent and five had transient inhibitors. The period during which samples were obtained was split into five time points (T) (Figure 2): at inhibitor detection (T0), from T0 to 30 days (T1), from day 31 to day 60 (T2), from 61 to 120 days (T3) and from 121 to 180 days (T4).

Laboratory methods

Anti-FVIII IgG subclasses were evaluated by the ELISA method described in detail in chapter I.

Statistical analysis

Associations of inhibitor persistence with anti-FVIII IgG subclasses and the main putative predictive factors (age at first treatment with FVIII concentrates [taken as a continuous variable], type of FVIII product [recombinant vs plasma-derived], number of exposure days [taken as a continuous variable], *F8* gene variation [null vs non-null], and highest inhibitor titer observed within the two months after first detection [high- vs low-titer]) were first estimated by univariate logistic regression analysis.

Table 1. Baseline characteristics of the patients included in Step 1 and Step 2 of the study.

	Anti-FVIII inhibitor				
	Step 1			Step 2	
Characteristic	Persistent (n = 31)	Transient (n = 12)	RR (95% CI)	Persistent (n = 9)	Transient (n = 5)
Age at first treatment, months	18 (8–29)	13 (8–15)	1·50 (1·00–2·98)	6 (4–30)	15 (9–15)
Age at inhibitor development, months	22 (13–33)	16 (11–18)	–	17 (8–32)	17 (11–18)
Number of exposure days	8 (5–10)	13 (10–17)	0·78 (0·52–3·93)	7 (5–8)	12 (11–19)
Type of FVIII product					
Plasma-derived	10 (32)	4 (33)	1·05 (0·23–4·23)	3 (33)	2 (40)
Recombinant	21 (68)	8 (67)	–	6 (67)	3 (60)
Inhibitor titre (BU)	19 (5–178)	2 (1–6)	–	32 (16–117)	2 (2–3)
High	21 (68)	3 (25)	1·66 (1·19–1·85)	9 (100)	1 (20)
Low	10 (32)	9 (75)	–	0 (–)	4 (80)
Family history of inhibitor					
No	25 (81)	11 (92)	–	8 (89)	4 (80)
Yes	4 (13)	0 (–)	–	1 (11)	0 (–)
NA	2 (6)	1 (8)	–	0 (–)	1 (20)
Type of FVIII mutation					
Null	28 (90)	10 (83)	1·47 (0·46–1·93)	8 (89)	5 (100)
Non-null	2 (7)	2 (17)	–	1 (11)	0 (–)
NA	1 (3)	0 (–)	–	0 (–)	0 (–)

RR, risk ratio; NA, not available; Continuous variables are expressed as medians (interquartile range), other variables are expressed as n (%).

Since odds ratios may lead to overestimation, relative risks (RR) and 95% confidence intervals (95% CI) were recalculated from the odds ratios according to Zhang and Yu.[64] Predictive factors that showed an association (RR lower limit of the 95% CI above 1), were then included in the multivariable analysis. Accordingly, predictive associations were evaluated with inhibitor persistence as the outcome.

First, a model for the prediction of inhibitor persistence based on previous data was presented. This model considered as predictor only high inhibitor titers (ROC curve of inhibitor titer adjusted for age at first treatment), so far reported in the literature as the only relevant risk factor associated with inhibitor persistence.[30,24,53,54] Then, we added into the model the predictor found from our analysis of Step 1 (i.e., number of anti-FVIII IgG subclasses present). The prediction performance was then assessed taking as outcome discrimination, that evaluates how well a model distinguishes between people who had or not the event (inhibitor persistence). In our analysis discrimination was assessed by means of the Harrell's C-index of concordance (a type of C-statistic), that indicates the probability that the model is able to assign a higher risk to a random case than to a random non-case. This was expressed as the area under the receiving operative characteristic (ROC) curve (AUC) with 95% CI, which plots "sensitivity" (true positive rate) against "1 – specificity" (false positive rate).

Results

Analysis of Step 1 (within 60 days of inhibitor detection)

Baseline characteristics of the 43 patients in Step 1 are shown in Table 1. Median age was 18 months (IQR: 12 to 29), the median inhibitor titer was 11 Bethesda IU (IQR: 3 to 127), and 24 (56%) were high-titer inhibitors (defined as titers of 5 or more Bethesda IU). Thirty-one of the 43 inhibitors (72%) were persistent, 12 (28%) transient. Among these 43 patients, the presence of only one IgG subclass (IgG1) was detected in three, two subclasses in 15, three subclasses in 13, and all four subclasses in 12 (Table 2). IgG1 was the only immunoglobulin G present in all the cases, thereby offering no information to distinguish transient from persistent inhibitors. Table 3 shows the various combinations of IgG subclasses based on the persistent/transient nature of the inhibitor.

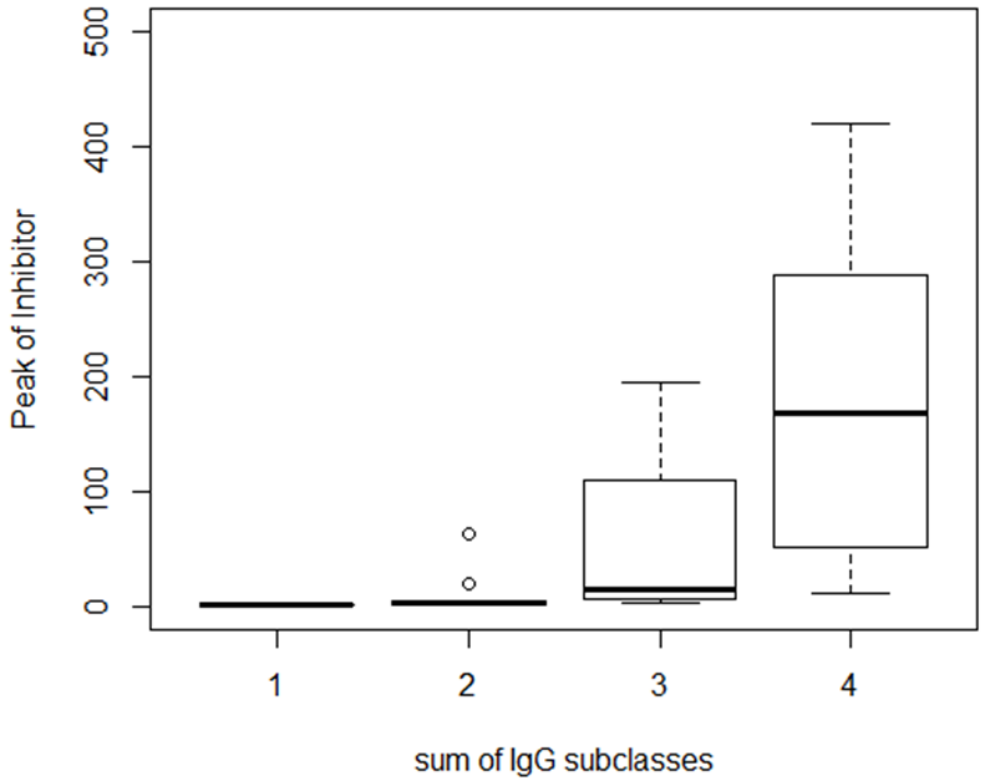
Table 2. IgG subclass combinations and anti-FVIII inhibitors of persistent and transient nature in Step 1.

	Anti-FVIII inhibitor	
IgG subclass combinations	**Persistent**	**Transient**
IgG1	1	2
IgG1 + IgG3	3	2
IgG1 + IgG4	5	5
IgG1 + IgG2 + IgG3	2	0
IgG1 + IgG3 + IgG4	9	2
IgG1 + IgG2 + IgG3 + IgG4	11	1
Total	**31**	**12**

The inhibitor titer was associated with the concomitant presence of all the IgG subclasses (Figure 3): patients with three or more subclasses showed more often high-titer inhibitors (22 of 25), whereas the three patients with only one IgG1 subclass had lower titers.

At univariate analysis inhibitor titer (RR 1.66 [95% CI: 1.19 to 1.85] for high-titer vs low-titer) and age at first FVIII treatment (RR 1.50 [95% CI: 1.00 to 2.98] for every six-month increase of age) were associated with inhibitor persistence (Table 1). No association was found for the source of FVIII (RR 1.05 [95% CI: 0.23 to 4.23]), number of exposure days (RR 0.78 [95% CI: 0.52 to 3.93] for every five exposure days), and type of mutation (RR 1.47 [95% CI: 0.46 to 1.93] for null vs non-null). At multivariable analysis, a high inhibitor titer remained associated with persistence (adjusted RR 1.67 [95% CI: 1.19 to 1.86] for high- vs low-titer). The likelihood of inhibitor persistence progressively increased with the number of IgG subclasses. In an age-adjusted analysis, the persistence RR was 1.71 [95% CI: 0.25 to 2.94] for patients with two IgG subclasses, 2.57 [95% CI: 0.73 to 3.01] for those with three, and 2.81 [95% CI: 1.15 to 3.02] for those with all four subclasses, all relative to patients with only one IgG subclass (IgG1) (Table 3).

Figure 3. Relationship between inhibitor peak titer and number of IgG subclasses. Each box plot represents the median value and interquartile range of the inhibitor peak titer, while the vertical bars represent the 2 SD. Open circles indicate outliers.



The Cochran-Armitage test for trend was applied to evaluate whether the increase of RR in parallel with the rising number of IgG subclasses is statistically significant. The test confirmed a significant linear trend in proportions (P = 0.006). Based on Table 3, for cases with one or two subclasses, the predictive value was 50% (9 of 18), for those with three or four subclasses 88% (22 of 25). The AUC value of the model with high inhibitor titer as predictor (adjusted for age at first treatment) was 0.78 [95% CI: 0.62 to 0.94] (Figure 4.A). The AUC of this model, with the number of IgG subclasses and adjusted for age at first treatment included as an additional predictor was 0.83 [95% CI: 0.69 to 0.97] (Figure 4.B).

Table 3. (A) Risk of inhibitor persistence according to the combination of IgG subclasses (Step 1). (B) Anti-FVIII IgG subclasses detection in the 14 patients* over 6 months following inhibitor development (Step 2).

Data are expressed as n (%) or relative risk (RR) [95% confidence interval (CI)]. *The 14 patients (nine with persistent inhibitors, five with transient inhibitors) became 13 for time points T3 and T4 due to limited plasma sample in the persistent inhibitor group as the nine patients with persistent inhibitors became eight for time points T3 and T4 due to limited plasma. †Calculated from odds ratios according to Zhang[64] and adjusted for age at first treatment.

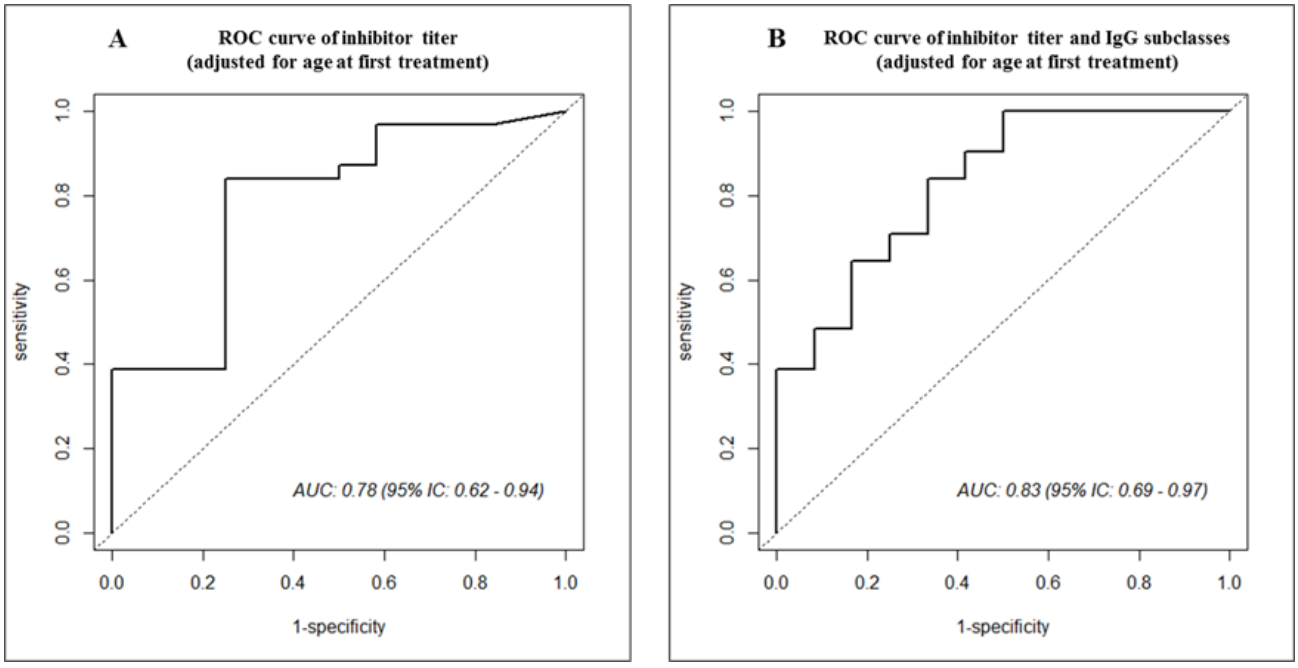
(A)

	Anti-FVIII inhibitor		
Combination of IgG subclasses	Persistent (n = 31)	Transient (n = 12)	RR (95% CI)†
1 subclass	1 (3)	2 (17)	1 (reference)
2 subclasses	8 (26)	7 (58)	1·71 (0·25–2·94)
3 subclasses	11 (35)	2 (17)	2·57 (0·73–3·01)
4 subclasses	11 (35)	1 (8)	2·81 (1·15–3·02)

(B)

	IgG1 positive/total		IgG2 positive/total		IgG3 positive/total		IgG4 positive/total	
Time point	Persistent	Transient	Persistent	Transient	Persistent	Transient	Persistent	Transient
T0	9/9 (100)	5/5 (100)	2/9 (22)	1/5 (20)	9/9 (100)	5/5 (100)	7/9 (78)	3/5 (60)
T1	9/9 (100)	5/5 (100)	5/9 (56)	1/5 (20)	9/9 (100)	3/5 (60)	8/9 (89)	4/5 (80)
T2	9/9 (100)	4/5 (80)	6/9 (67)	1/5 (20)	9/9 (100)	4/5 (80)	9/9 (100)	4/5 (80)
T3	8/8 (100)	5/5 (100)	7/8 (88)	1/5 (20)	6/8 (75)	4/5 (80)	8/8 (100)	5/5 (100)
T4	8/8 (100)	4/5 (80)	4/8 (50)	0/5 (0)	5/8 (63)	2/5 (40)	8/8 (100)	5/5 (100)

Figure 4. ROC curves of the predictive model for inhibitor persistence containing inhibitor titer only (A), and inhibitor titer + number of IgG subclasses (B). The broken diagonal line represents the situation of predictive uncertainty, corresponding to AUC=0.50.

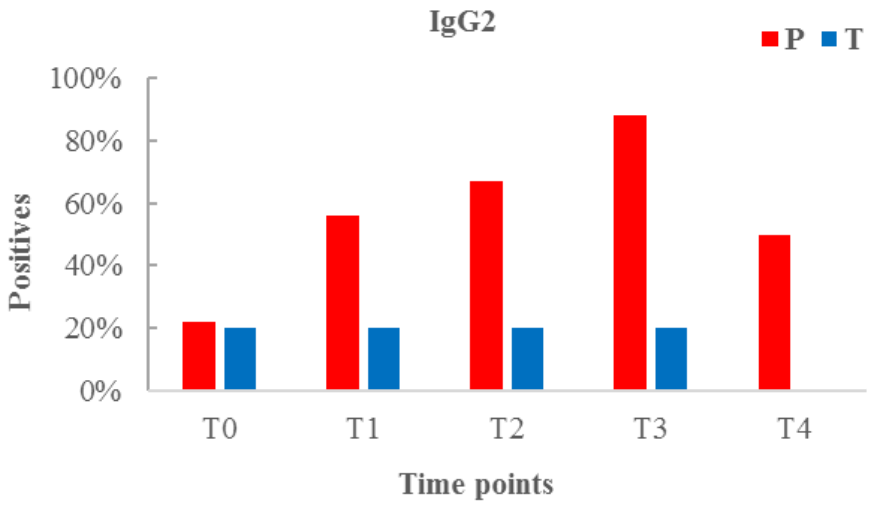


Analysis of Step 2 (within six months of inhibitor detection)

Baseline characteristics of the 14 patients in Step 2 are shown in Table 1. Table 3 shows that IgG1 was the only IgG subclass invariably present throughout the sequential time points in patients with both transient and persistent inhibitors. IgG3 presence in both the persistent and transient inhibitor patient subgroups decreased from 100% at the time of inhibitor detection to 40% and 63% at the last time point in the transient and persistent subgroups, respectively. IgG4 was increasingly present with time in patients in both subgroups, starting with 60% and 78% in transient and persistent subgroups and ending with 100% by the last time point. IgG2 was the only anti-FVIII IgG subclass that showed a clear difference between the transient and persistent subgroups. In the transient subgroup it remained present in only 20% of patients from inhibitor detection until T3 and was not

detectable in any patient at the last time point, whereas in the persistent subgroup it increased from 22% at inhibitor detection to 88% at the last time point T3 (Figure 5).

Figure 5. Anti-FVIII IgG2 subclass detection in the 14 patients*, over 6 months following inhibitor development. P: persistent. T: transient. *: The 14 subjects became 13 for time points T3 and T4 due to limited plasma sample in the persistent inhibitor group.



The IgG2 subclass profiles resulting from the two SIPPET *post-hoc* longitudinal analyses are summarized in Figure 6 (results from chapter I and II). Given the number of patients with IgG2 who developed a persistent inhibitor (12/19), that of patients with IgG2 who ended up with a transient inhibitor (2/15) and that of patients with persistent inhibitors who had no IgG2 (7/19), the odds ratio of having IgG2 and ending up with a persistent inhibitor was calculated (Table 4). An OR 95% CI of 11.43 [1.92 – 64.54] was obtained from these two studies, suggesting that the odds of having IgG2 and developing a persistent inhibitor is up to 11 times higher than that of having IgG2 and a transient inhibitor.

Figure 6. IgG2 prevalence in the two assessments of the IgG subclass signatures in two different subsets of patients from the SIPPET study, with their respective study designs and transient/persistent inhibitor cohorts.

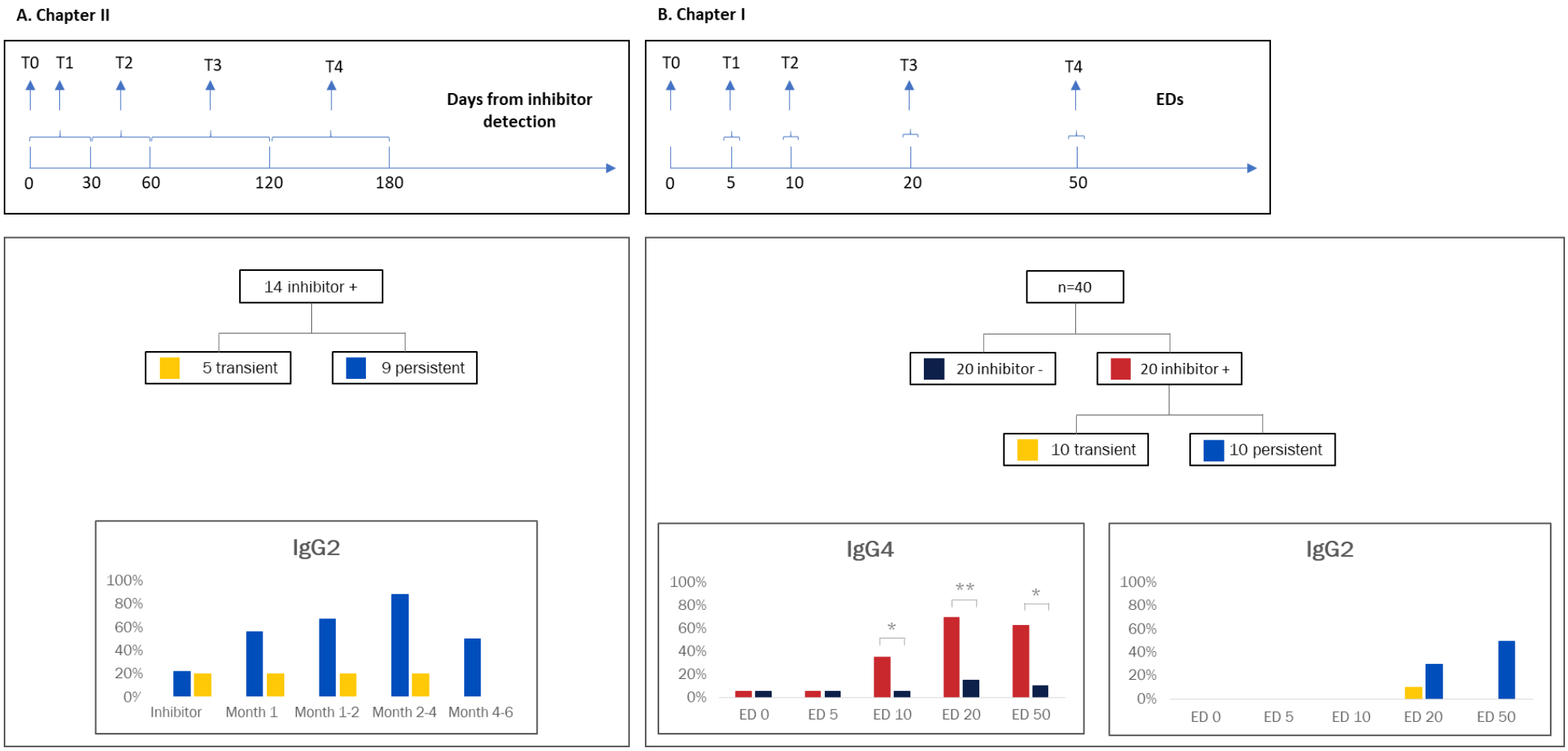


Table 4. Odds of having IgG2 (at any time point) and having a persistent inhibitor calculated on the basis of our 2 studies.

	Transient inhibitor (and IgG2+)	**Persistent inhibitor (and IgG2+)**
Chapter II	5 (1/5)	9 (8/9)
Chapter I	10 (1/10)	10 (4/10)
	15	19

	Transient inhibitor (no event)	Persistent inhibitor (event)	OR [95% CI]
IgG2+ (exposure)	2	12	11.43 [1.92 – 64.54]
IgG2- (no exposure)	13	7	
	15	19	

Discussion

Due to the scarcity of longitudinal studies on inhibitor development, the multicausal phenomenon of inhibitor persistence remains unclear.[37,44,51,53] The most robust previous finding was that a high inhibitor titer at the time of first detection is associated with a high likelihood of persistence.[30,41-45]

With this background and gaps of knowledge, we present herewith the results of a study exploring both the early and late stages of inhibitor development. The main difference between the present study and the previous one was the time window considered and consequently the target population: while in the first study we looked at a population that was inhibitor free at baseline and ended up with 50% inhibitor patients at the last time point analyzed, the focus in the present study was only on inhibitor patients, looking at the post inhibitor development phase. The Step 1 analysis focused on the first 2 months post inhibitor development and Step 2 on the dynamic pattern of the anti-FVIII IgG subclasses over time, through a longitudinal analysis on the 6 months post inhibitor development, i.e., the time frame that defines inhibitor persistence.

Step 1 showed that while IgG1 had no predictive value for inhibitor persistence, the likelihood of inhibitor persistence progressively increased with the degree of heterogeneity of the IgG subclass profile (i.e., the concomitant presence of more than one anti-FVIII IgG subclass). We saw an association between a high inhibitor titer at onset and inhibitor persistence. This was in line with our previous observation (chapter I, step 1, part II) where in 90% of patients with persistent inhibitors, individual subclass profiles revealed the presence of at least 3 different subclasses in the time span of 50 EDs, suggesting a correlation between a higher combination of different IgG subclasses with inhibitor persistence. Step 2 results showed that IgG1, IgG3, and IgG4 did not

distinguish patients with transient from those with persistent inhibitors. IgG2 was the only IgG subclass that showed a differential pattern, because the relative abundancy of this subclass increased gradually in the persistent group, supporting once again IgG2 as the only anti-FVIII IgG subclass that distinguishes persistent inhibitors from transient ones, late in the course of FVIII exposure (around 20 EDs according to chapter I results) but early after inhibitor development (chapter II), as hallmark of persistent inhibitors. We saw a peak of IgG2 prevalence in the persistent inhibitor population between 2-4 months post inhibitor development.

All in all, in the SIPPET cohort, taken together the analyses of chapters I and II, the association of IgG2 with persistent inhibitors was an observation made on a total of 19 persistent inhibitors, compared to 15 transient ones. These two studies show for the first time that IgG2 may be a hallmark of inhibitor persistence, with the odds of having IgG2 and developing a persistent inhibitor being up to 11 times higher than that of having IgG2 and a transient inhibitor. However, the relevant role of IgG2 in this process remains to be established. A comprehensive functional study, depicting the upstream cytokines modulating the switch to IgG2, alongside the downstream effectors induced by this subclass, could clarify the mechanisms undertaking continued immunoreactivity against FVIII.

Limitations of this study are the absence of information on how patients were treated post inhibitor formation, because SIPPET was not designed to collect data on patient treatments after inhibitor development, as well as the limited sample size. Nevertheless, this is so far the largest longitudinal study designed to predict inhibitor persistence with samples collected in the time frame of the early post inhibitor period.

Conclusion

Our longitudinal *post-hoc* SIPPET studies on anti-FVIII IgG subclasses show for the first time that IgG2 is the only subclass that may be considered a hallmark of inhibitor persistence, detectable as early as 2-4 months after development in most patients who develop it, with the inhibitor in those carrying this subclass 11 times more likely to become persistent. However, the assessment of this hallmark in persistent inhibitors of similar cohorts is warranted in order to externally validate this observation, allowing to help optimize candidate selection to identify those patients with bad prognosis (likelihood of inhibitor persistence) benefitting from early ITI or alternative personalized therapeutic approaches.

In the following step we continued our investigation to study the inhibitor development phenomenon from an epidemiological point of view. We designed an observational retrospective cohort study to measure the risk of inhibitor development, this time in patients already treated for at least 50 EDs (PTPs), upon a change of treatment approach (chapter III).

Chapter III

Inhibitor development upon switch from plasma-derived to recombinant factor VIII in previously untreated patients with severe hemophilia A: the PUP-SWITCH study

Abstract

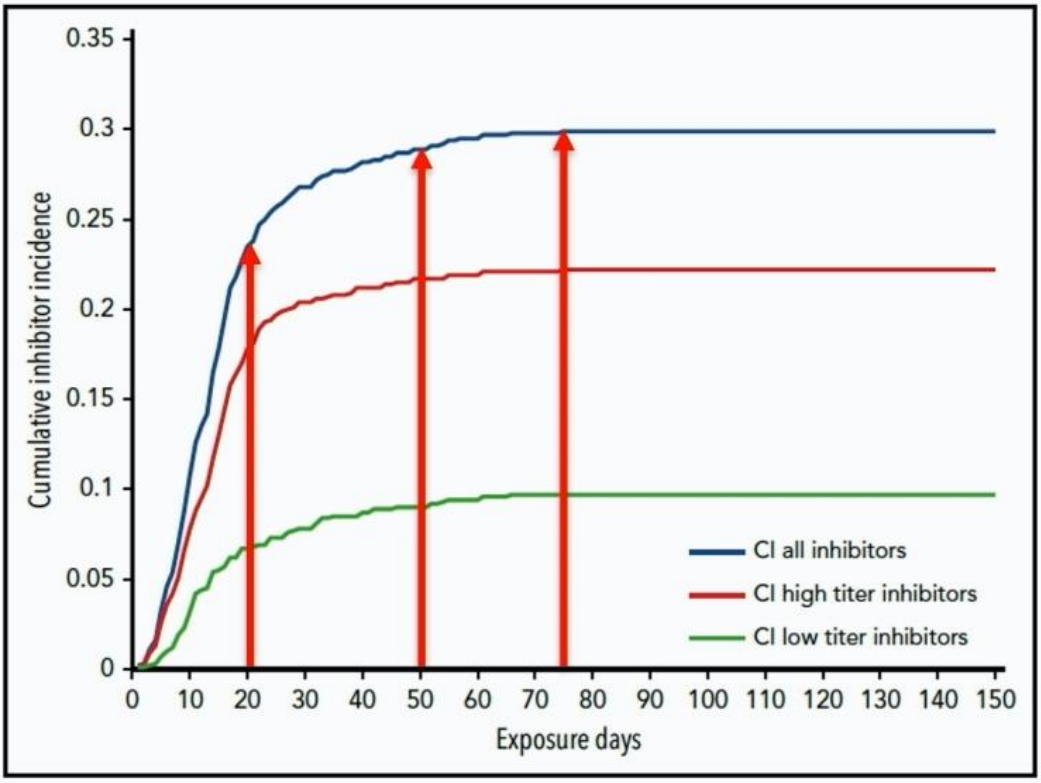
In 2016, the SIPPET study reported that in PUPs with severe hemophilia A the choice of pdFVIII products in the first 50 EDs is associated with a significantly lower incidence of inhibitors compared to rFVIII products. However, the choice of FVIII product class after this high-risk period has been subject to debate. It has been established that switching products in multi-transfused (> 50 EDs) PTPs does not affect the risk of inhibitor development which becomes approximately 100 times lower in these patients. We aimed to investigate whether a switch from pdFVIII to rFVIII in PUPs after 50 EDs would follow the expected low-risk pattern of inhibitor incidence as reported in PTPs, or a novel peak of inhibitors would appear due to the change of product class. We designed a survey to investigate the rate of novel inhibitors after switching PUPs between 50 and 150 EDs. Centers who had already switched PUPs under 6 years old with severe hemophilia A according to this strategy were invited to participate. Data of 97 patients were analyzed, among which 87 satisfied all eligibility criteria. One patient developed an inhibitor after the switch. Such incidence rate is no higher than the expected risk of neo-immunogenicity after 50 EDs in the absence of a switch. In summary, results of the 87 patients included in the PUP-SWITCH showed for the first time that a switch from pdFVIII to rFVIII after 50 EDs is safe, with a minimal risk of inhibitor incidence close to 1%.

Introduction

In the last 20 years, several observational studies and systematic reviews have suggested a higher rate of inhibitor incidence with use of rFVIII.[15,65-68] However, for a long time there was insufficient evidence available to claim superiority of one class of products compared to the other. The SIPPET, the first randomized clinical trial to definitively settle this issue showed that during the first 50 EDs, the cumulative incidence of all inhibitors was 27% with pdFVIII and 45% with rFVIII.[26] In other words, recombinant products proved to be almost twice more immunogenic than plasma-derived products in PUPs. Numerous studies have shown that patients treated with at least 50 EDs, also referred to as previously treated patients (PTPs), have a significantly lower risk of inhibitor development which becomes approximately 100 times lower compared to PUPs going through the initial exposure phase.[32,70,71] It is believed that switching products in these multi-transfused patients or PTPs does not affect the risk of inhibitor development.[72]

It is unclear whether switching among products of the same class, or between product classes is associated with a clinically relevant increase in the risk of inhibitor development, or would the low-risk pattern of PTPs remain valid despite such switch. The PedNet, the largest ongoing prospective cohort study over 20 years on an estimated population of 4000 PUPs with severe hemophilia A, has aimed to investigate the risk periods for inhibitor development until 1000 EDs. In 2019, they reported that in over 1000 PUPs, cumulative inhibitor incidence was 28.9% at 50 EDs and 29.9% at 75 EDs (Figure 1).[73]

Figure 1. Kaplan Meier curve of 1038 patients with severe HA followed from ED 1 to ED 150 in the PedNet study. (van den Berg et al. Blood 2019)



Further data on inhibitors in PTPs have been reported from national registries, international registries, post-marketing surveillance studies, registration trials and meta-analyses.[15,28,32,56,74-86] Taken together, the incidence of inhibitors in hemophilia A PTPs has been estimated at about 2 per 1000 patient-years but high variability is reported between product brands.[56,69,86] However, little is known about the neo-immunogenicity in these PTPs as they have not been thoroughly or systematically investigated. Indeed, inhibitors in PTPs are usually attributed to loss of tolerance to FVIII. The observed rate of inhibitors in this population, at different phases of treatment, has been proposed by the Scientific and Standardization Committee (SSC) of the International Society on Thrombosis and Haemostasis (ISTH).[87-89] The most recent definition proposed that is still being

revised and drafted to be published in the coming year, was discussed at the ISTH 2022 Congress (Table 1).

Table 1. Proposal for new nomenclature based on exposure day status, ISTH 2022 Guy Young, SSC session on FVIII, FIX and Rare Coagulation Disorders.

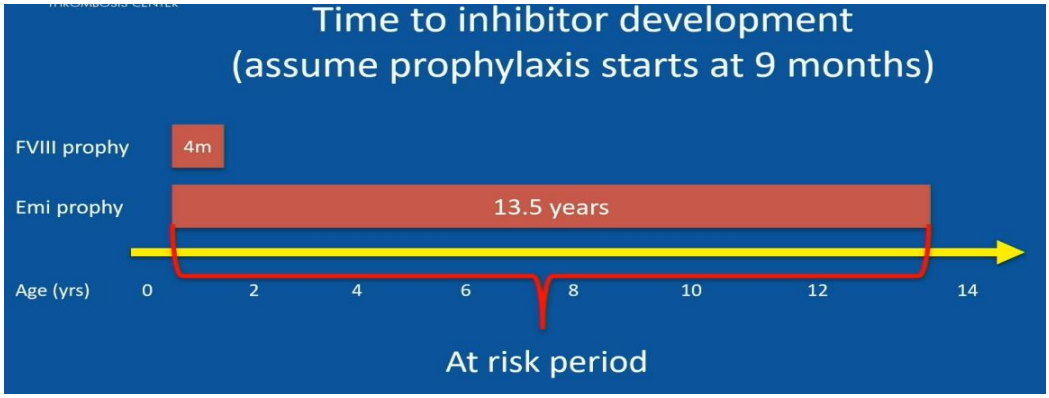
Factor Exposure Status (exposure days)					
Exposure day range	(0 EDs)	(1-5 EDs)	(6-20)	(21-50 EDs)	(>50 EDs)
New nomenclature	PUP	Early MTP	Mid MTP	Late MTP	PTP
Cumulative risk (%)	0	36 (for 10 EDs)	79	89	99

A treatment strategy consisting of pdFVIII use in the early high-risk period followed by a switch to rFVIII, would combine the benefits of both types of treatment, particularly for those who do not wish to be treated with pdFVIII indefinitely. A reason that may prompt to consider such switch in these patients is the current availability of extended half-life rFVIII products, which allows to reduce by 20-30% the number of yearly intravenous injections, thus improving patients' quality of life. Another reason that may encourage to choose rFVIII is the perception that these products may be safer than those derived from human plasma, in terms of transmission of blood-borne infectious agents that might escape the current robust methods of viral inactivation. A perception that although may be considered unrealistic by some given that the risk of such transmission is very low, remains among the worries of parents of newly diagnosed hemophiliacs, who may have seen family members go through the disastrous years of HIV/HCV outbreaks.

With this background, the strategy of "initial 50 EDs with pdFVIII followed by a switch to rFVIII (standard or extended half-life)" may become a standard of care if the incidence of inhibitors using this approach is not unacceptably higher than the known incidence rate in PTPs after 50 EDs. Many patients world-wide have been treated according to this strategy, notably after the SIPPET results. However, to date, no unique centralized registry to collect the data on these patients has been created.[90] Given this gap, we designed the PUP-SWITCH observational study to make use of the existing data on patients who have been treated according to local standard practice, in line with the aforementioned switch strategy.

The importance of this research question in the case of a patient going through FVIII replacement therapy is quite evident. In addition, also for patients not receiving FVIII replacement therapy, notably those in treatment with the FVIII mimicking bispecific antibody, emicizumab, the switch research question remains a critical contemporary issue to investigate, because emicizumab alone cannot assure correct hemostasis during an acute hemorrhagic event.[91-93] The same holds true also for new non-factor therapies, based on re-balancing coagulation by reducing anti-thrombin (AT) or inhibiting Tissue Factor Pathway Inhibitor (TFPI), drugs now in clinical trial testing. Patients being treated with all these non-factor drugs, require FVIII infusion for the management of breakthrough bleeds and surgical procedures. While reaching the inhibitor development stage in about 4 months (by 20-50 EDs) on FVIII prophylaxis, in patients on emicizumab prophylaxis, inhibitor development is delayed by more than 13 years (Figure 2).[94]

Figure 2. Model for time to inhibitor development proposed by Patel et al. J Med Econ. 2019. schematised by Guy Young at the ISTH 2022 Congress.



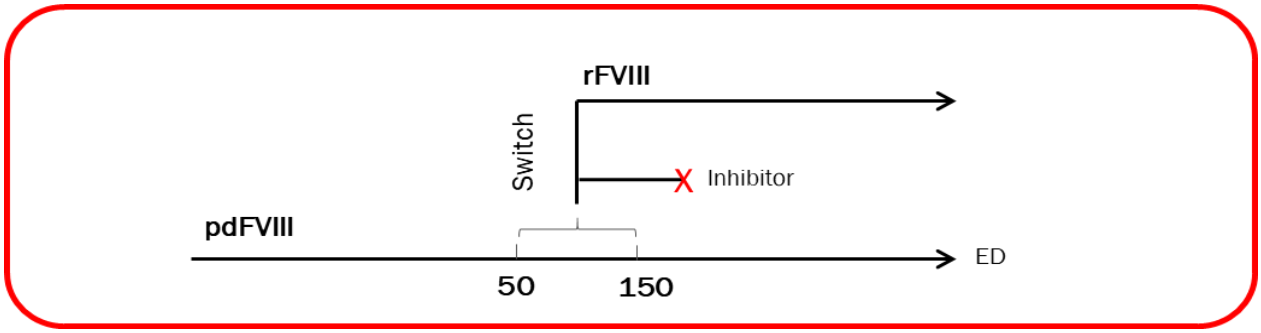
In short, the evaluation of the switch strategy remains a scientifically relevant issue, perhaps now more than ever, with the availability of these non-factor therapies, given the postponing of FVIII exposures, thus, postponing the inhibitor risk, delayed to an older age when it might coincide with a breakthrough bleed or a planned surgery, complicating hemorrhagic management.

Materials and Methods

Study design

PUP-SWITCH is an international, multicenter, observational cohort study on inhibitor occurrence in PUPs/MTPs with severe hemophilia A, aged under 6 years at initiation of FVIII replacement therapy. Those who did not develop an inhibitor on a pdFVIII during a first treatment phase of at least 50 and maximum 150 EDs, and were subsequently exposed to a rFVIII product, for at least 50 EDs after the switch or 2 years of treatment (Figure 3) were included in the study. Apart from the switch, any change in the type of product was reason for non-eligibility. Per protocol, patients treated with a pdFVIII prophylactic regimen who started prophylaxis within the 10th ED were includable. The project received ethical approval from Milan Area 2 Committee.

Figure 3. PUP-SWITCH study design.



Study Outcomes

The primary endpoint was inhibitor development after the switch. The secondary endpoints were the modality of inhibitor development, by measuring outcomes such as number of EDs, titer at

onset, peak titer, persistence/transience of the inhibitor, clinical factors such as age at first treatment, family history of hemophilia and of the inhibitor, and *F8* gene mutation.

Rationale for number of patients

In 2015, DiMichele and colleagues provided recommendation and guidelines on clinical study design for the evaluation of inhibitor development in PUPs and PTPs with severe hemophilia A.[89] Accordingly, immunological theories of neo-immunogenicity in different ED phases, provide an estimate of the cohort size needed to study the inhibitor incidence in the corresponding phase, prefixing a maximum tolerable risk. Such studies are primarily sized to rule out a greater than acceptable risk of FVIII product immunogenicity in PTPs.[95] In this guideline paper, two main phases were defined for the expected inhibitor risk:

• Phase 1 - Epidemic phase (0 – 50 EDs): inhibitor incidence of 30%, with the measured outcome being cumulative incidence (events/people).[96,97]

• Phase 2 - Endemic phase (50 – 150 EDs): inhibitor rate of 4/100 person-years, with the measured outcome being a "higher than acceptable rate" to be excluded (/person-years).[96]

In the second phase, sample size is dependent on both the predefined rate of inhibitor development to be excluded and the person-time accrued in the study. One or less inhibitor event in 80 subjects is required to rule out a 6.8% inhibitor cumulative incidence over 50 EDs, based on the upper level of the 95% confidence interval in a Poisson distribution.[97] Accordingly, by setting the rule-out risk at 7%, supposing 1 inhibitor incidence and considering a non-evaluable rate of 10%, a sample size of

88 was calculated to carry out the study (Table 2). A total of 80 eligible patients (Table 2) were needed to obtain a result with an 80% power.

Table 2. Sample size requirements proposed by DiMichele et al. JTH 2015 in the epidemic (phase 1) and endemic (phase 2).

Phase 1			
	Observed number of inhibitors allowed		
	0	1	3
Cumulative incidence ruled out*			
4	91	137	178
5	72	110	142
6	60	91	118
7	51	78	101
8	45	68	88
9	40	60	78

Phase 2			
	Observed numbers of inhibitors allowed		
	0	1 ←	2
Incidence rate (per 100 person-yrs)			
2	185 [16%]†	279 [23%]	362 [30%]
3	123 [29%]	186 [45%]	241 [57%]
4	93 [40%]	140 [59%]	181 [73%]
5	74 [48%]	112 [69%]	145 [82%]
6	62 [54%]	93 [76%]	121 [88%]
→ 7	53 [59%]	80 [81%]	104 [91%]
8	47 [63%]	70 [84%]	91 [94%]

*One-sided $\alpha = .025$; exact binomial model used according to current regulatory consensus.
†Power assuming a true 1% inhibitor incidence.

Data collection and management

Upon the patients' informed consent, demographic data, clinical profiles, laboratory data and therapeutic regimens were extracted from patient charts and recorded in a survey designed on the ISTH REDCap platform, in form of a protected electronic database.[98]

Laboratory Methods

Centers reported to have used the one-stage Bethesda assay to measure the inhibitor.[99] The frequency of inhibitor testing was at the discretion of each individual center.

Statistical Analysis

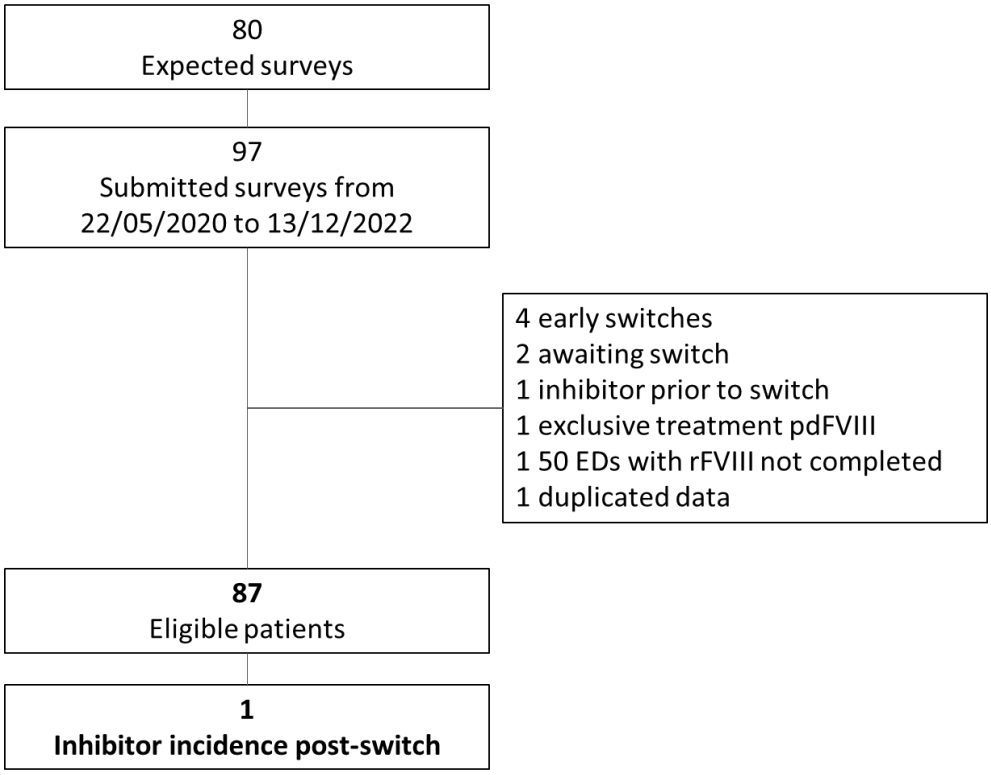
Incidence rates and cumulative incidences were calculated by person-years methods and time-to-event (survival) techniques, with error margins based on Poisson and binomial distributions. Incidence rates were calculated as the number of inhibitor incidences divided by total follow-up time in years and EDs, multiplied by 1000 to obtain a rate in "cases per 1000 person-years or person-EDs".

Results

Study population

A total of 97 surveys were submitted to the PUP-SWITCH survey platform from 22/05/2020 to 13/12/2022, of which 87 were eligible. Ten were excluded from the present analysis for various non-eligibility reasons. Twelve Turkish, 3 Iranian, 1 German and 1 Egyptian center participated in the study. In the 87 eligible patients, 1 developed an inhibitor after the switch (Figure 4).

Figure 4. Flowchart.



We asked for an updated status of the patient data submitted between 22/05/2020 and 25/02/2021 (patients 1 to 49) with a special focus on number of rFVIII EDs since the switch (up to now, 2023), and whether the inhibitor status changed. Data of the patients included in PUP-SWITCH in the

second half of 2022 (patients 49 to 87) were considered “up to date”. Baseline characteristics, ethnic origins, treatment characteristics, products used as well as switch features and follow-up times of the patient population are shown in Table 3. The prevalence of null mutations in our study population was 49%, with the most common mutation being intron 22 inversion (20%), followed by non-sense mutation (13%), frameshift (12%) and large deletion/duplication (6%). Fifteen percent of patients had non-null mutations that comprised missense (10%), splice site mutation (2%), and polymorphism (1%). One patient was reported to have no mutation and 30 patients did have molecular data available based on routine practice. Thirty-eight percent of patients had a positive family history of hemophilia and 4% a positive family history of inhibitors (Table 3.A).

Table 3. A. Baseline characteristics of the PUP-SWITCH population in terms of *F8* mutation, family history of hemophilia and of the inhibitor.

Baseline characteristics	Inhibitor - (N=86)	Inhibitor + (N=1)	All (N=87)
Type of F8 mutation			
Frameshift	11 (12.8%)	0 (0%)	11 (12.6%)
Intron 22 inversion	17 (19.8%)	0 (0%)	17 (19.5%)
Large deletion	2 (2.3%)	1 (100%)	3 (3.4%)
Large duplication	2 (2.3%)	0 (0%)	2 (2.3%)
Missense	9 (10.5%)	0 (0%)	9 (10.3%)
None	1 (1.2%)	0 (0%)	1 (1.1%)
Nonsense	11 (12.8%)	0 (0%)	11 (12.6%)
Polymorphism	1 (1.2%)	0 (0%)	1 (1.1%)
Splice site	2 (2.3%)	0 (0%)	2 (2.3%)
Unknown	30 (34.9%)	0 (0%)	30 (34.5%)
Family history of hemophilia			
No	53 (61.6%)	0 (0%)	53 (60.9%)
Yes	33 (38.4%)	1 (100%)	34 (39.1%)
Family history of inhibitor			
No	83 (96.5%)	0 (0%)	83 (95.4%)
Yes	3 (3.5%)	1 (100%)	4 (4.6%)

With regards to ethnic backgrounds, all patients were of white ancestry (Middle Eastern and European). They were 29% of Iranian, 26% of Egyptian, 25% of Turkish and 20% of German ethnic background (Table 3.B).

Table 3. B. Baseline characteristics of the PUP-SWITCH population in terms of ethnic background.

Origins	Inhibitor - (N=86)	Inhibitor + (N=1)	All (N=87)
City			
Ankara	4 (4.7%)	0 (0%)	4 (4.6%)
Cairo	22 (25.6%)	1 (100%)	23 (26.4%)
Denizli	2 (2.3%)	0 (0%)	2 (2.3%)
Duisburg	17 (19.8%)	0 (0%)	17 (19.5%)
Esfahan	2 (2.3%)	0 (0%)	2 (2.3%)
Gaziantep	1 (1.2%)	0 (0%)	1 (1.1%)
Istanbul	4 (4.7%)	0 (0%)	4 (4.6%)
Izmir	8 (9.3%)	0 (0%)	8 (9.2%)
Kocaeli	1 (1.2%)	0 (0%)	1 (1.1%)
Samsun	2 (2.3%)	0 (0%)	2 (2.3%)
Shiraz	1 (1.2%)	0 (0%)	1 (1.1%)
Tehran	22 (25.6%)	0 (0%)	22 (25.3%)
Country			
Egypt	22 (25.6%)	1 (100%)	23 (26.4%)
Germany	17 (19.8%)	0 (0%)	17 (19.5%)
Iran	25 (29.1%)	0 (0%)	25 (28.7%)
Turkey	22 (25.6%)	0 (0%)	22 (25.3%)

Seventy-two percent were PUP and 28% were MTP. The reason for first treatment was bleeding in 58% of cases, prophylaxis in 33% and surgery (mainly circumcision) in 9%. The median age at first treatment was 10 months (Table 3.C).

Table 3. C. Treatment characteristics of the PUP-SWITCH population in terms of previous treatment, reason for first treatment and age at first treatment.

Treatment characteristics	Inhibitor - (N=86)	Inhibitor + (N=1)	All (N=87)
Previous treatment			
MTP	24 (27.9%)	1 (100%)	25 (28.7%)
PUP	62 (72.1%)	0 (0%)	62 (71.3%)
Reason for first treatment			
Bleeding	50 (58.1%)	1 (100%)	51 (58.6%)
Prophylaxis	28 (32.6%)	0 (0%)	28 (32.2%)
Surgery	8 (9.3%)	0 (0%)	8 (9.2%)
Age at first FVIII infusion (months)			
Median [Q1, Q3]	10 [7, 14]	2 [2, 2]	10 [7, 14]

With regards to rFVIII products used after the switch, Xyntha was the most common product used in 32% of patients, followed by NovoEight (19%), Nuwiq (17%), SaFacto (14%), ReFacto (10%), Advate (5%), Kogenate (2%) and Kovaltry (1%) (Table 3.D).

The popularity of Xyntha was mainly because it was used in a subset of Iranian patients, during the time when it was the only product that could still be imported to Iran. The four rFVIII products most commonly used in this patient cohort, Xyntha, NovoEight, Nuwiq and ReFacto, all have a B-domain deleted formulation, with the advantage of not requiring the use of blood-derived products such as human serum albumin, minimizing the risk of transmitting blood-borne pathogens, and increasing the manufacturing yield of the product yet maintaining the in vitro/in vivo functionality intact. [100,101] Among them, Nuwiq is the only human cell line-derived rFVIII in the market while all other rFVIII products are animal cell line-derived (from Chinese Hamster Ovary (CHO) cells and Baby Hamster Kidney (BHK) cells) and has demonstrated low immunogenicity.[102]

SaFacto is an Iranian rFVIII used commonly nation-wide and currently the only rFVIII available in Iran due to imposed sanctions that have everlastingly weighed more on the people of Iran rather than on the incompetent regime that has isolated the country, limiting its exchange with the outside world; pharmaceutical drug import is not an exception in this.

Safacto has a B-domain-deleted fabrication, made from CHO cell line, that has been compared to Xyntha in a small randomized trial on 33 patients. Results suggested similar safety and efficacy using either one of the two products. The rate of immunogenicity of SaFacto, however, remains unclear.[103,104]

Table 3. D. Products used in the PUP-SWITCH population in the pdFVIII and rFVIIII treatment phase.

Products used	Inhibitor - (N=86)	Inhibitor + (N=1)	All (N=87)
pdFVIII product			
Beriate	5 (5.8%)	0 (0%)	5 (5.7%)
Emoclot	5 (5.8%)	0 (0%)	5 (5.7%)
Factane	8 (9.3%)	0 (0%)	8 (9.2%)
Haemate	1 (1.2%)	0 (0%)	1 (1.1%)
Haemoctin	3 (3.5%)	0 (0%)	3 (3.4%)
Immunate	13 (15.1%)	0 (0%)	13 (14.9%)
Koate	10 (11.6%)	1 (100%)	11 (12.6%)
Octanate	25 (29.1%)	0 (0%)	25 (28.7%)
Unknown	16 (18.6%)	0 (0%)	16 (18.4%)
rFVIII product			
Advate	4 (4.7%)	0 (0%)	4 (4.6%)
Kogenate	2 (2.3%)	0 (0%)	2 (2.3%)
Kovaltry	1 (1.2%)	0 (0%)	1 (1.1%)
NovoEight	15 (17.4%)	1 (100%)	16 (18.4%)
Nuwiq	15 (17.4%)	0 (0%)	15 (17.2%)
ReFacto	9 (10.5%)	0 (0%)	9 (10.3%)
SaFacto	12 (14.0%)	0 (0%)	12 (13.8%)
Xyntha	28 (32.6%)	0 (0%)	28 (32.2%)

Patients were switched at a median of 60 EDs and had received a median of 200 EDs after the switch, corresponding to a median follow-up of 1.3 years (data on 84 patients) (Table 3.E). Pie charts of the *F8* mutations as well as pdFVIII and rFVIII products used in these 87 patients is shown in Figures 5 and 6.

Table 3. E. Switch characteristics and follow-up features of the PUP-SWITCH population.

Switch and follow-up post-switch	Inhibitor - (N=86)	Inhibitor + (N=1)	All (N=87)
ED of switch			
Median [Q1, Q3]	61.5 [51, 73]	50 [50, 50]	60 [51, 73]
EDs of rFVIII post-switch			
Median [Q1, Q3]	204.5 [108.75, 409]	20 [20, 20]	200 [108, 408]
Total EDs (pdFVIII + rFVIII)			
Median [Q1, Q3]	272.5 [177, 510.25]	70 [70, 70]	270 [176, 504.5]
Time of observation post-switch (years)			
Median [Q1, Q3]	1.33 [0.98, 4.27]	0.15 [0.15, 0.15]	1.33 [0.97, 4.19]
Missing	3 (3.5%)	0 (0%)	3 (3.4%)

Figure 5. *F8* mutation chart representation.

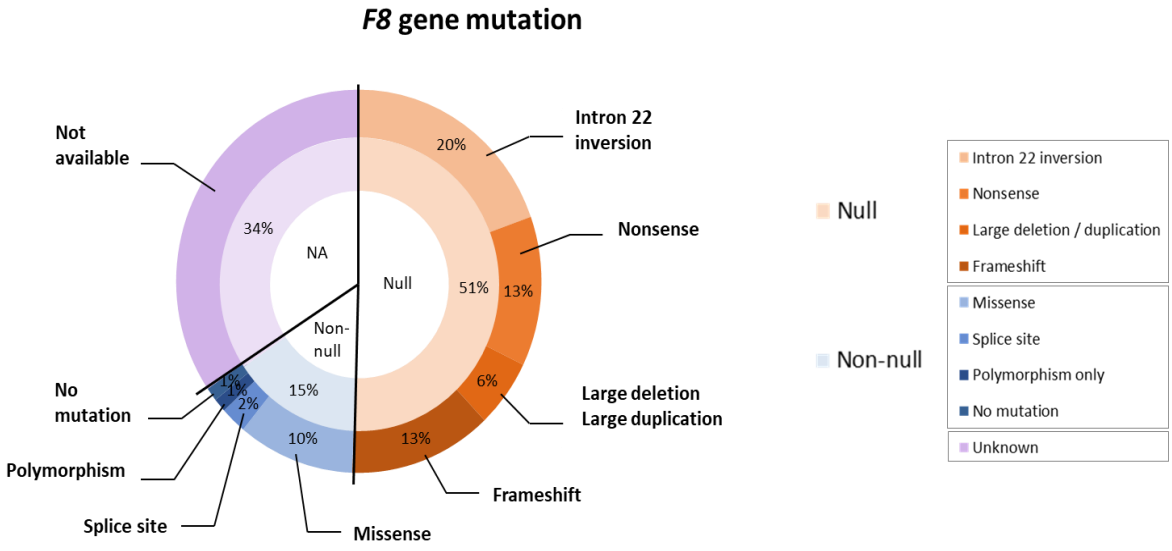
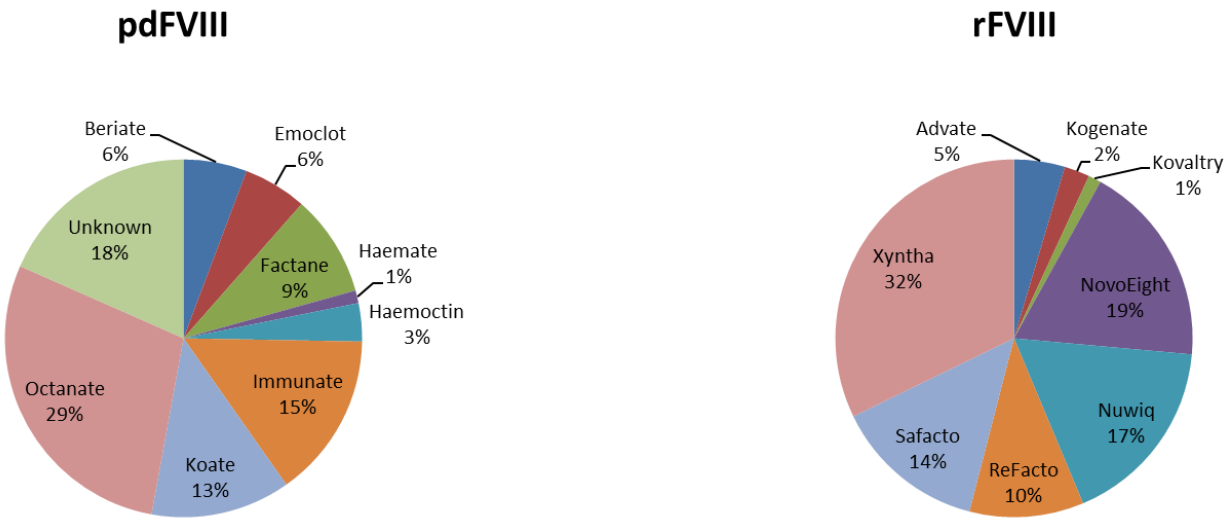


Figure 6. pdFVIII and rFVIII product choices in the PUP-SWITCH population.



Inhibitor incidence

The patient who developed an inhibitor was switched to NovoEight at ED 50 and developed an inhibitor by ED 70. At onset, the inhibitor was low-titer (3 BU/ml), reached peak (8 BU/ml) by 4 weeks, and was undetectable at 9 weeks post onset (transient). Regarding the baseline characteristics of this patient, he had a null *F8* mutation (large deletion), with a family history of hemophilia and of the inhibitor. He was an MTP who had received 3 infusions with cryoprecipitate prior to initiation of FVIII replacement therapy with Koate to treat a traumatic bleeding event at 2 months of age.

Years at risk calculation was as follows, according to the different time lapses reported (Table 4): considering a follow-up fixed at 50 EDs for all 87 patients, the total time at risk for the PUP-SWITCH cohort was 4320 EDs, considering the full observation time available for patients, time at risk was 25488 EDs, and finally, 224.15 years considering calendar dates for the 84 patients who

had this data available (for 3 patients this data was not provided). Incidence rates and cumulative incidence are reported in Table 4.

Table 4. Incidence rates using different time measures and cumulative incidence.

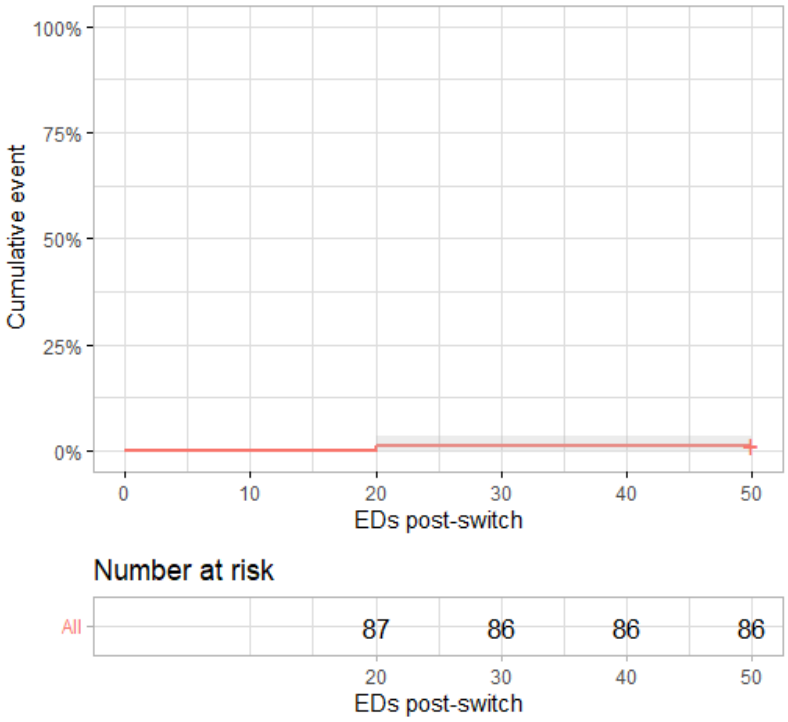
	Fixing 50 EDs for all patients	Complete follow-up available for each patient	Complete follow-up available for each patient
n	87	87	84
Time measure	EDs	EDs	Years
Time at risk	4320	25488	224.15
Incidence Rate	23.15 in 1000 person-100 EDs	3.92 in 1000 person-100 EDs	4.46 in 1000 person-years
95% Confidence Interval	1.26 – 164.29	0.55 – 27.85	0.63 – 31.53
Cumulative Incidence	1.15 %		

The incidence rate considering a follow-up fixed at 50 EDs for all 87 patients was 23.15 in 100000 people in 1 ED, therefore a rate of 23.15 per 1000 person-100 EDs (95% CI 3.26 – 164.29). Taking the full observation time available for patients, the incidence rate calculated was 3.92 in 100000 people in 1 ED, therefore a rate of 3.92 in 1000 person-100 EDs (95% CI 0.55 – 27.85). Taking calendar dates and not EDs as measure of time for the full observation time available for 84 patients, the incidence rate calculated was 4.46 per 1000 person-years (95% CI 0.63 – 31.53). Cumulative incidence was 1.15 %. Survival curves were traced with cumulative events on the Y

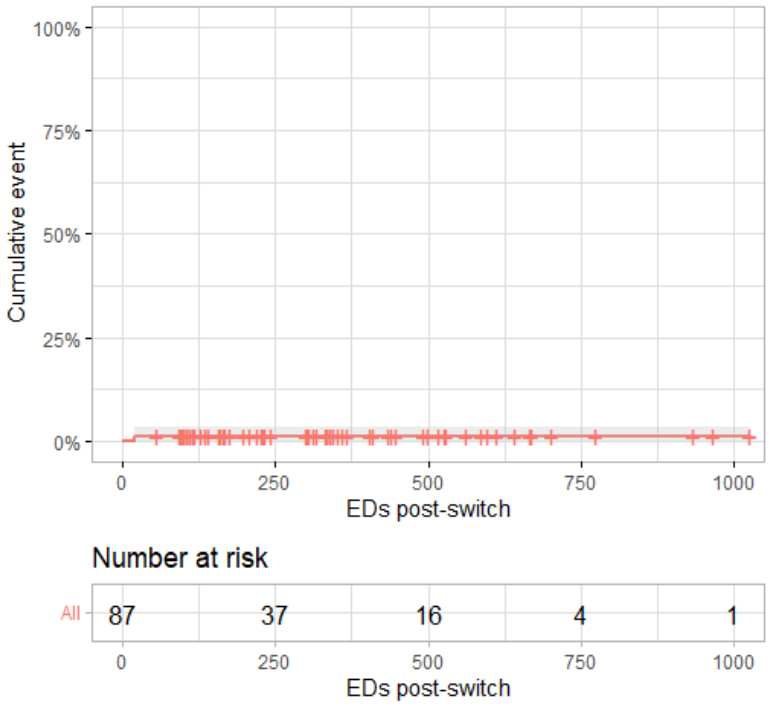
axis and EDs post-switch on the X axis: the latter was traced both considering a follow-up fixed at 50 EDs (Figure 7.A) and considering the full observation time available for each patient included (Figure 7.B).

Figure 7. Cumulative event curves with follow-up fixed at 50 EDs for all patients (A) and considering the true complete follow-up available for patients (B).

A.



B.



Discussion

One of the questions that remained unanswered after the SIPPET was whether once the patient overcomes the early high-risk phase of exposure to FVIII (initial 50 EDs), the immune tolerance that has settled would remain despite a potential change of therapeutic approach. In this study we chose to explore the rate of inhibitor development upon a switch from the "safer" product class (pdFVIII) to the more immunogenic product class (rFVIII) once the vulnerable phase has elapsed. Such "switch" would allow to combine the benefits of both product classes: pdFVIII (lower inhibitor risk in the early phase) and rFVIII (extended half-life products that have led to easier prophylaxis management and lower annual factor use, zero viral transmission risk and unlimited availability thanks to their independence on blood donations). The PUP-SWITCH results yielded no additional risk of inhibitor development upon the switch. Only one patient developed an inhibitor after the switch. Given the single inhibitor incidence, association analysis to investigate the putative factors potentially associated with late inhibitor development in this patient is not possible. However, descriptive analysis of Table 3 by comparison of this inhibitor patient with the total population, highlight the positive family history of inhibitor, a rare condition present in 6% of the cohort. Regarding the genetic background of this cohort, null mutations were present at a prevalence of 49%. A prevalence of 80% has been reported in the literature for null mutations in such population.[105-107] Considering a 35% of our patient population for whom molecular analysis was not performed, such discrepancy was expected.

Cumulative incidence was 1.15% and inhibitor incidence rate was less than 4 in 1000 person-100 EDs, corresponding to 4.46 in 1000 person-years. The inhibitor rate after 50 EDs in various meta-

analyses and prospective cohort studies has been reported to be 2.06 per 1000 person-years (95% CI 1.06 – 4.01) in the latest meta-analysis available in the literature, by Hassan and colleagues in 2019.[108] The latter took into consideration rFVIII products only and included also moderate hemophilia A patients. Kempton and colleagues reported an incidence rate of 2.14 in 1000 person-years in their 2006 study, a retrospective cohort study, where they considered patients treated with both pdFVIII and rFVIII.[77] The McMillan et al. paper published in 1988 was taken as reference of inhibitor rate in PTPs before meta-analysis results were available, where they reported a rate of 8 per 1000 person-years.[109,110] Overall, reports of inhibitor incidence in PTPs have yielded difficultly comparable results. Optimal design of clinical trials on immunogenicity notably in PTPs is hampered by an incomplete understanding of the precise nature of interactions between a FVIII product and the recipient's immune system. Despite the availability of various single reports and meta-analyses, the number of patients who develop inhibitors after 50 EDs is too low to adequately evaluate risk factors for inhibitor development in the PTP population. Nonetheless, aware of the short observation time, variable between patients, and a small cohort, our overall data corroborate with the reports in the literature despite its wide 95% CI.

Kempton and colleagues hypothesized in their 2006 paper that there is a baseline rate of inhibitor development independent of new antigen exposure (such as change of product), suggesting together with Xi et al. in 2013 and Kim & You in 2019 that there are different mechanisms of inhibitor development in PTPs compared with PUPs; in PUPs perhaps more reliable on the genetic background and treatment-related factors, in PTPs more associated with non-genetic treatment-unrelated factors that could nonetheless stimulate the immune system leading to *de novo* inhibitor

development.[69,77,111] Presumably, more non-patient-oriented factors may impinge neo-immunogenicity in patients who have achieved immune tolerance to FVIII, being them at higher risk of exposure to adult-life occurrences such as infection, surgery, and cancer, which may break down the established immune tolerance. Data from the United Kingdom Haemophilia Centre Doctors' Organisation (UKHCDO) in 2011 supported this possibility, showing a second peak of inhibitor development in patients around their 60s.[74] The latter is the real-life evidence of the bimodal risk model proposed by the Kempton group in 2006 where they suggested a bimodal distribution for inhibitor incidence: with increasing age the risk of inhibitor occurrence could rise again.[74,77,108] In the latter theory, immunological ignorance is not easily distinguishable from active tolerance. Consequently, when a PTP develops an inhibitor after switching to a different product, an immunological break in pre-existing tolerance cannot be distinguished from product-associated neo-immunogenicity.[112]

The extremely low incidence rate of inhibitors in PTPs makes it difficult to perform association analyses, if not through large prospective multicenter registries, warranted for the homogeneous and bias-limited reporting of such data over several decades. Some examples of the latter are PedNet (including PUPs born between 2000 and 2030, followed up until their 18th birthday, expected to reach 4000 participants)[113,114], FranceCoag (national French registry, including patients since 2000, 22 years of follow-up and 1047 participants up to 2017)[115,116], UKHCDO (ongoing national UK registry, since 1978)[117], and EUHASS (European-scale registry of adverse event (such as inhibitor development) reporting, ongoing since 2008)[118] that would allow to capture such rare events.

Among the limitations of this study, we can point out the inherent drawbacks of observational studies that suffer from bias and confounding, non-homogeneous follow-up times, the variability of products used, and therapeutic regimens adopted, as well as the heterogeneous frequency of inhibitor testing which may have led to under-detection of the inhibitor incidence. Furthermore, shorter follow-up is associated with an incidence rate erroneously higher and longer follow-up with a rate that is lower.

Despite so, to date PUP-SWITCH is the only registry designed to evaluate the safety of a product class switch in PUPs with severe hemophilia A transitioning to become PTPs. Expanding this registry to also include patients in prophylaxis with emicizumab and other non-factor therapies could be a future perspective.

Conclusion

PUP-SWITCH showed that a switch from pdFVIII to rFVIII after 50 EDs is safe and not associated with an additional risk of inhibitor development. This strategy of starting FVIII exposure with plasma-derived products then switching to recombinant has the potential to become the standard of care for PUPs on FVIII prophylaxis and perhaps even in the era of non-replacement therapies, for patients requiring FVIII in the occasion of breakthrough events. However, future studies are needed investigate such hypothesis and further narrow down the switch window to evaluate the safety of an even earlier switch, before 50 EDs notably in children on non-replacement therapies in whom reaching 50 EDs takes several years.

Chapter IV

Thesis conclusion

Considerable efforts in the past decades have led to the manufacturing of novel hemophilia drugs that have improved the quality of life among hemophiliacs through enhancement of therapeutic efficacy, less frequent administrations, joint health preservation, and a life expectancy comparable to the general population. Despite so, the development of inhibitors still remains one of the most burdensome complications, the main challenge in the treatment of hemophilia. Inhibitor development is a multifactorial process; some risk factors have been identified and confirmed by most studies published in the literature while other factors remain putative candidates reported to play a role. Providing a better understanding of the different mechanisms underlying the peculiar immunogenicity of products with standardized approaches, over long follow-up times, through real-world data, on comparable patient populations is of extreme importance. Currently available new extended half-life FVIII products in clinical practice have proven their efficacy, however there is still scarce information on the rate of inhibitor development in PUPs, MTPs, and PTPs in an age-stratified manner to assess systematically the bimodal pattern of inhibitor incidence risk in the lifespan of a patient: the genetic- and treatment-related factors in PUPs and the lifestyle-related factors in mature PTPs where the danger theory has been suggested to play a crucial role, potentially capable of breaking the long-developed tolerance to FVIII.

Moreover, new non-replacement therapies (anti-TFPI antibody, bispecific antibodies, siRNA targeting antithrombin and SerpinPC) are currently being evaluated for routine prophylaxis in patients with and without inhibitors. Patients with inhibitors in treatment with anticoagulant quenchers, subcutaneous FVIII mimetic molecules and gene therapy, would still remain vulnerable and difficult to make therapeutic choices for, because during breakthrough bleeding episodes and surgical management, they are exposed to a thrombotic risk upon use of bypassing agents (APCC (Feiba) and rFVIIa (NovoSeven)) when combined with emicizumab and anticoagulant quenchers. On the other hand, given the short half-life of these agents and thus the need of multiple, very closely spaced intravenous injections, this subset of patients remains at higher risk of morbidity and mortality. Thus, notwithstanding the availability of a wide spectrum of non-factor therapies with interesting features, the inhibitor development complication remains a critical challenge for the treatment of the hemophilias, notably hemophilia A, as shown in the supplementary Table 1.

Considering the gaps of knowledge, this thesis tried to provide essentially two recommendations based on the obtained evidence:

1) Upon validation of this data, IgG2 could be a good candidate as inhibitor persistence predictor early after inhibitor detection, suggesting an anti-IgG2 subclass ELISA test worth adding to the usual Bethesda test during the follow-up, notably between 2-4 months after development. The detection of this subclass could be considered a marker of bad prognosis (persistent inhibitor), comforting the clinician in his/her choice of initiating ITI with more confidence, as the patient is seemingly not prone to tolerizing the inhibitor endogenously on his own.
2) A switch after 50 EDs from pdFVIII to rFVIII is immunologically safe thus could be considered as a strategy to more confidently treat patients in need to factor, both in a FVIII replacement therapy context and in a novel therapy one, allowing to combine the advantageous features of both classes of FVIII products, each at their respective beneficial phase.

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Sigaud M, Turea V, Wu JKM, Vdovin V, Pavlova A, Jansen M, Belyanskaya L, Walter O, Knaub S, Neufeld EJ. Immunogenicity, efficacy and safety of Nuwiq® (human-cl rhFVIII) in previously untreated patients with severe haemophilia A-Interim results from the NuProtect Study. Haemophilia. 2018 Mar;24(2):211-220. doi: 10.1111/hae.13320. Epub 2017 Aug 16. PMID: 28815880.

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Supplementary Figures and Tables

Chapter I

Figure 7. Schematic representation of the IgG subclass profile for the 20 patients who developed inhibitors: patient 1

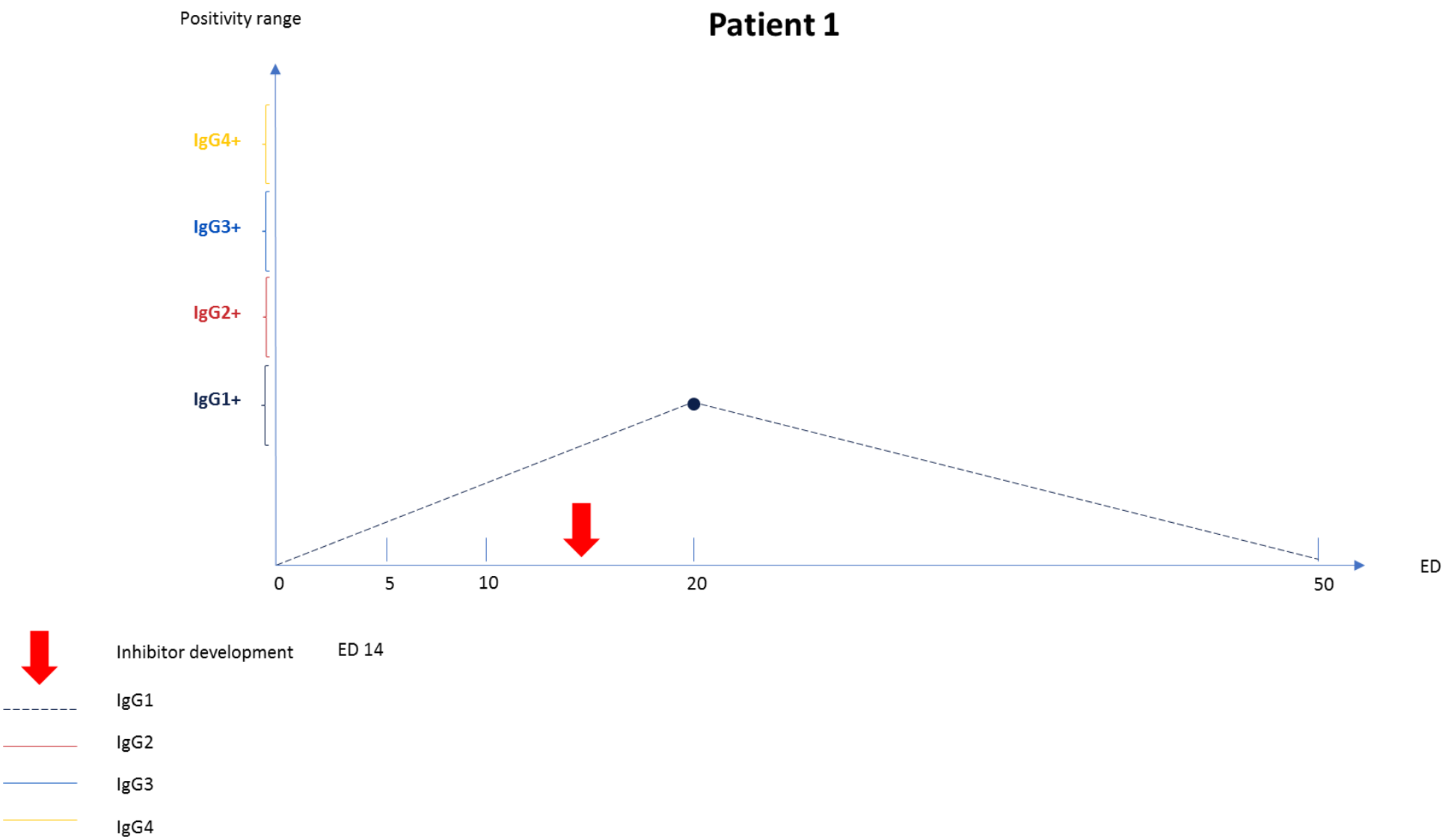


Figure 7. Schematic representation of the IgG subclass profile for the 20 patients who developed inhibitors: patient 2

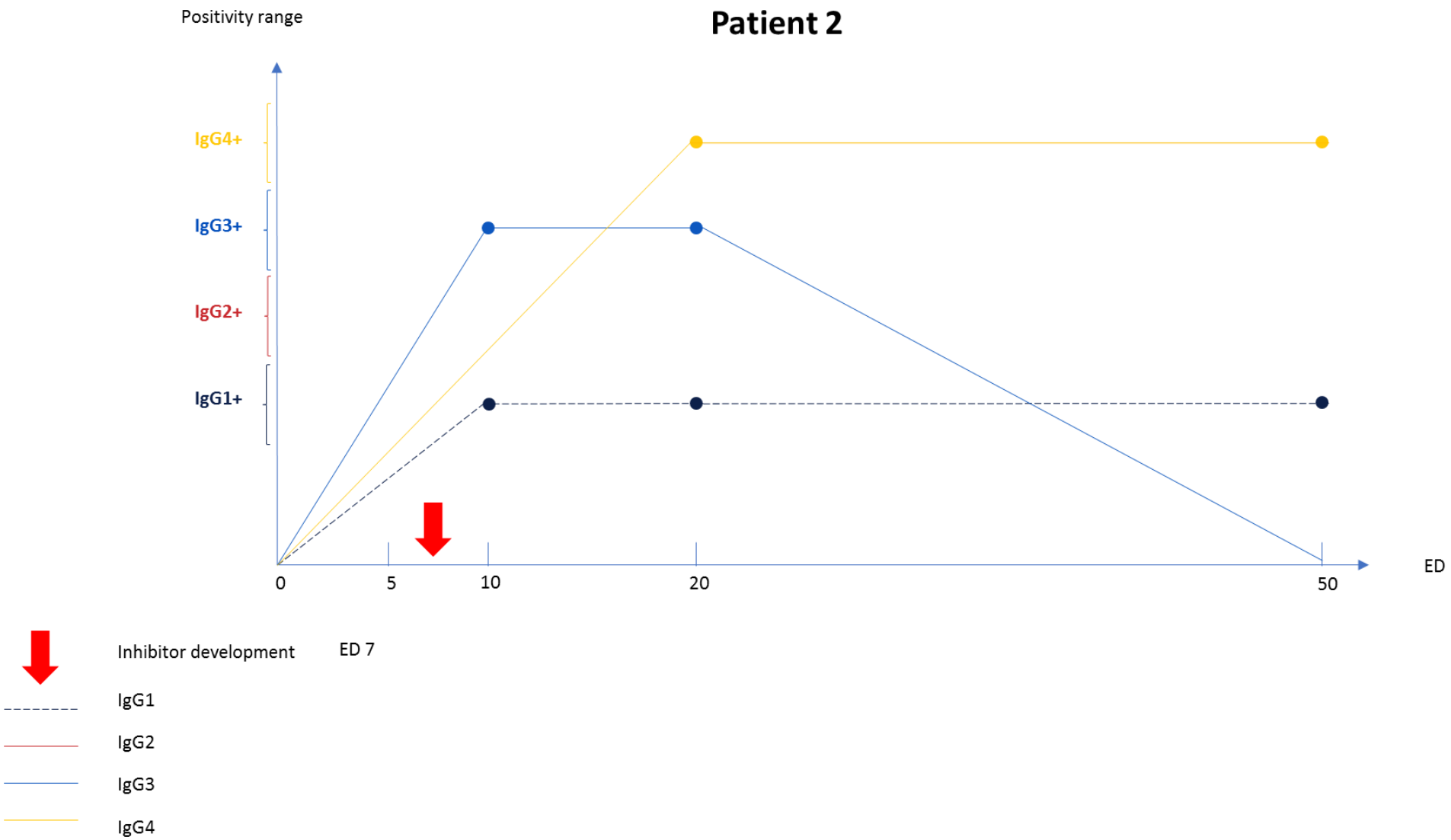


Figure 7. Schematic representation of the IgG subclass profile for the 20 patients who developed inhibitors: patient 3

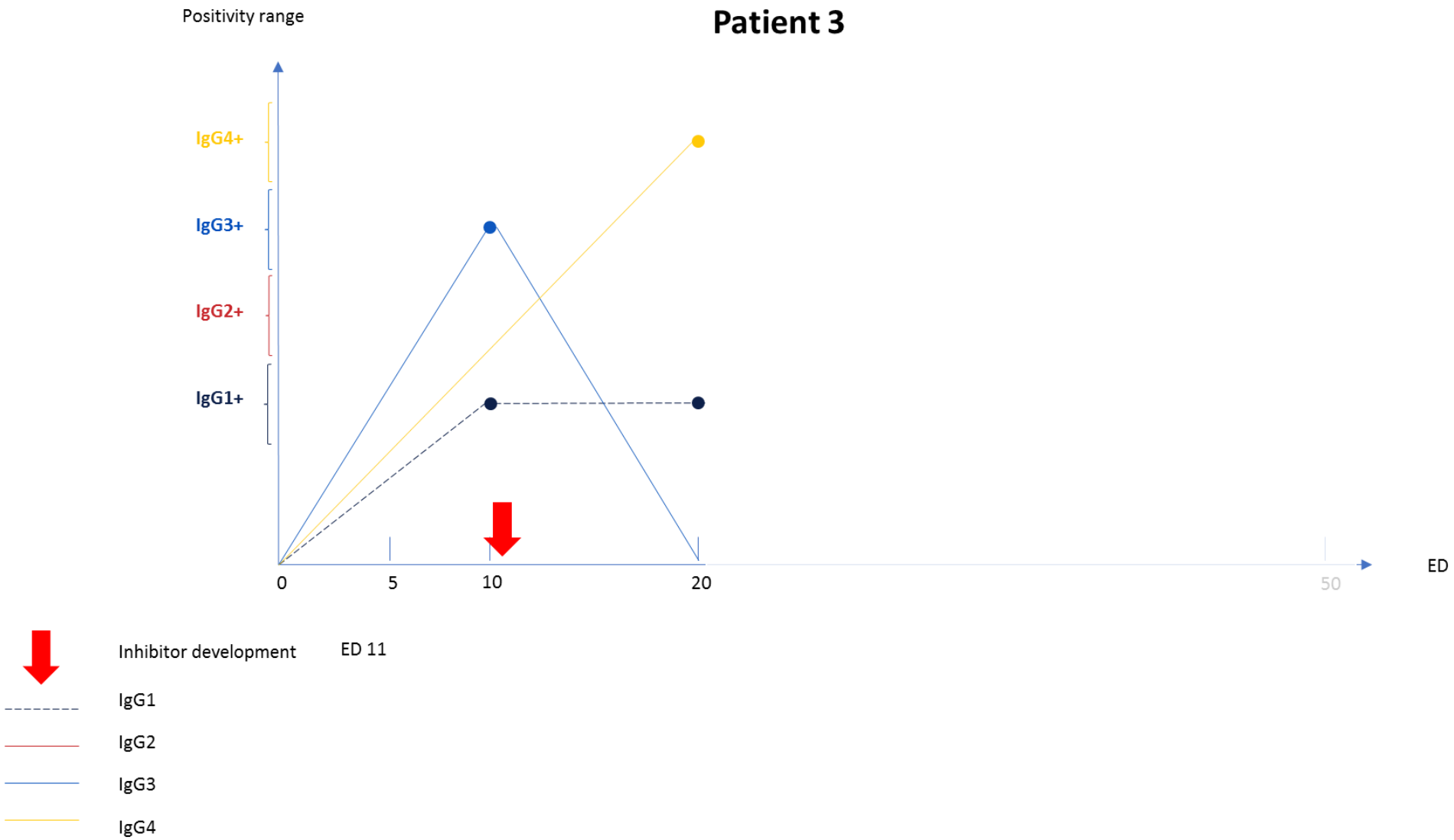


Figure 7. Schematic representation of the IgG subclass profile for the 20 patients who developed inhibitors: patient 4

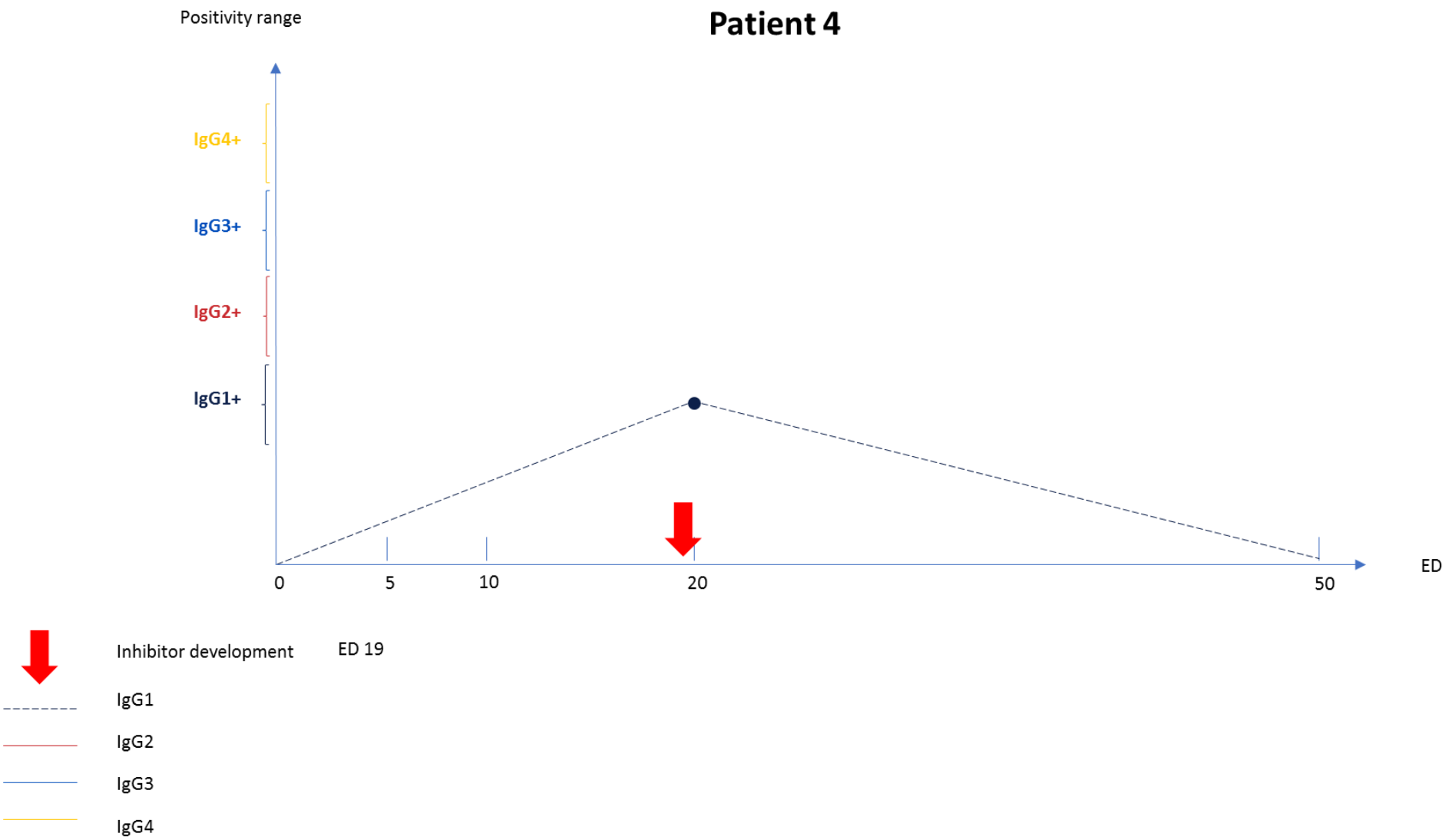


Figure 7. Schematic representation of the IgG subclass profile for the 20 patients who developed inhibitors: patient 5

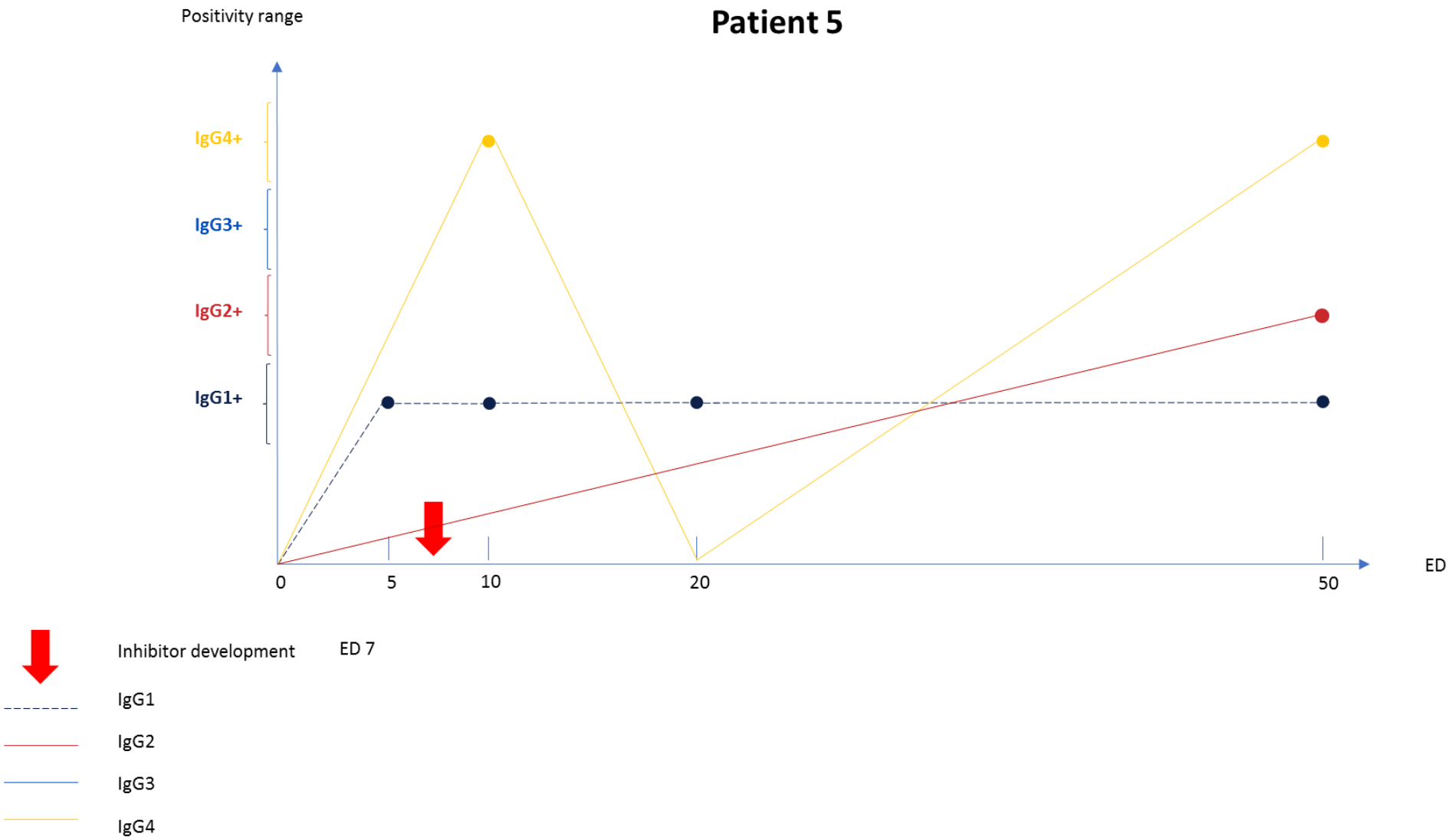
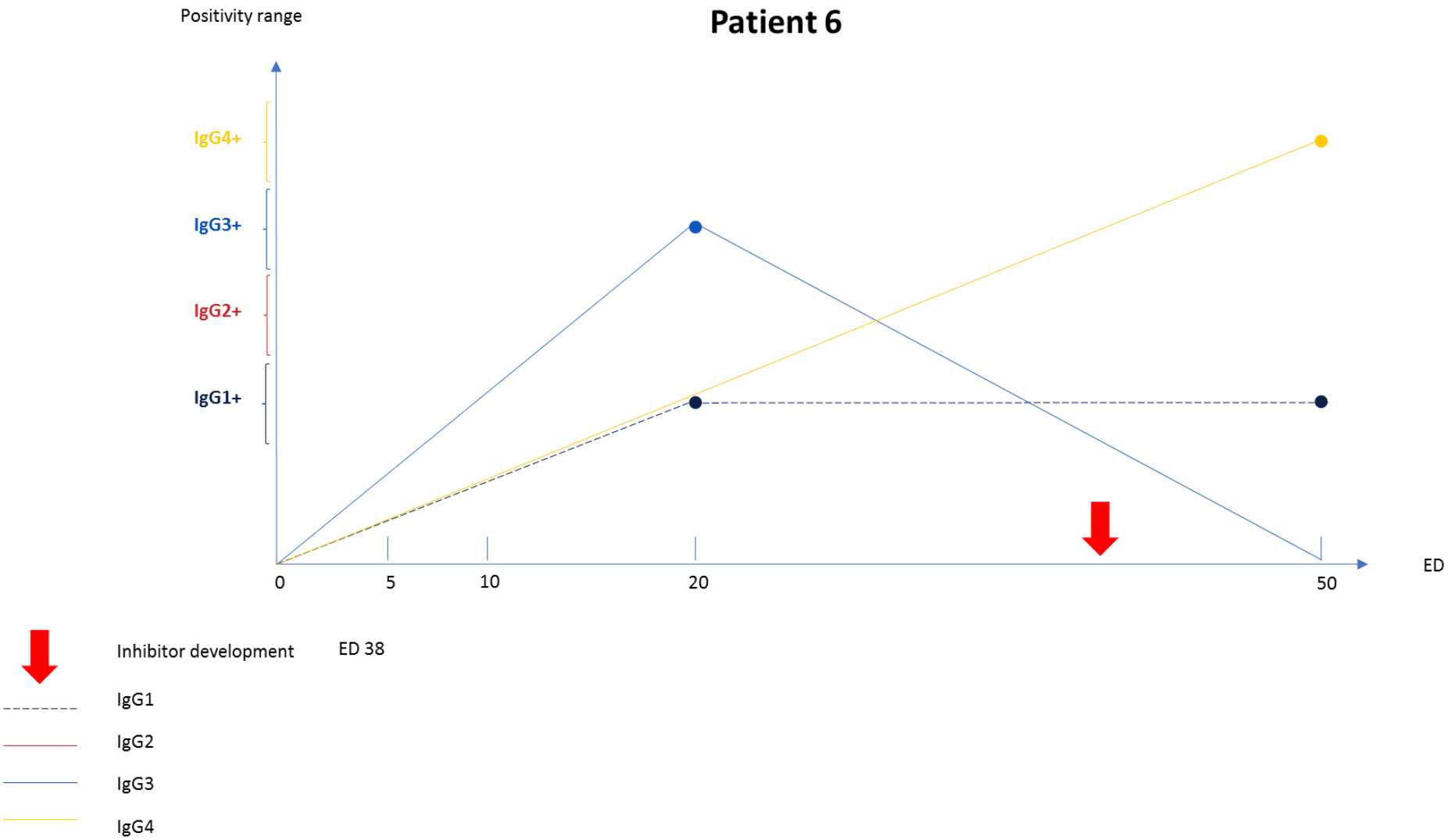


Figure 7. Schematic representation of the IgG subclass profile for the 20 patients who developed inhibitors: patient 6



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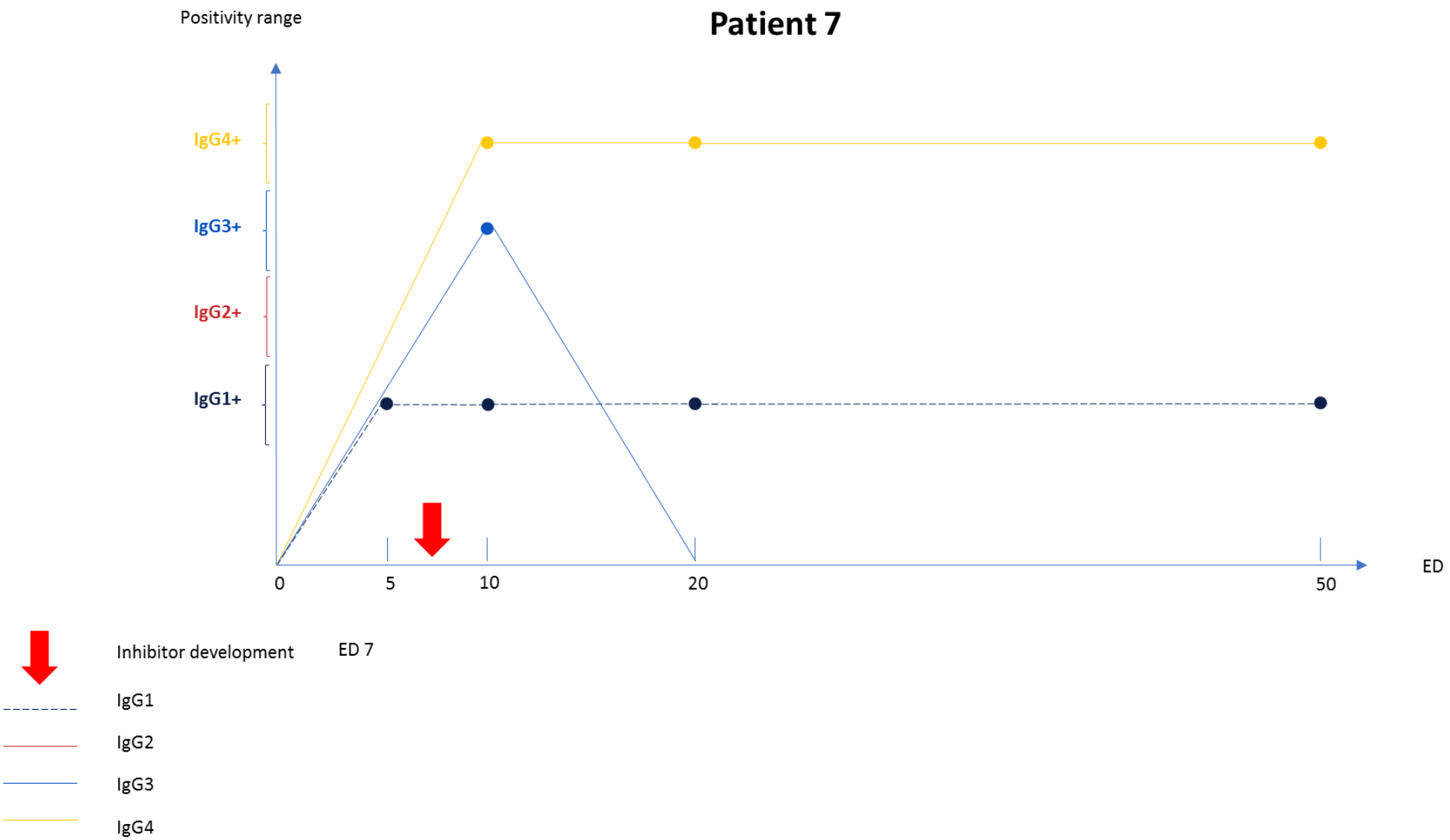

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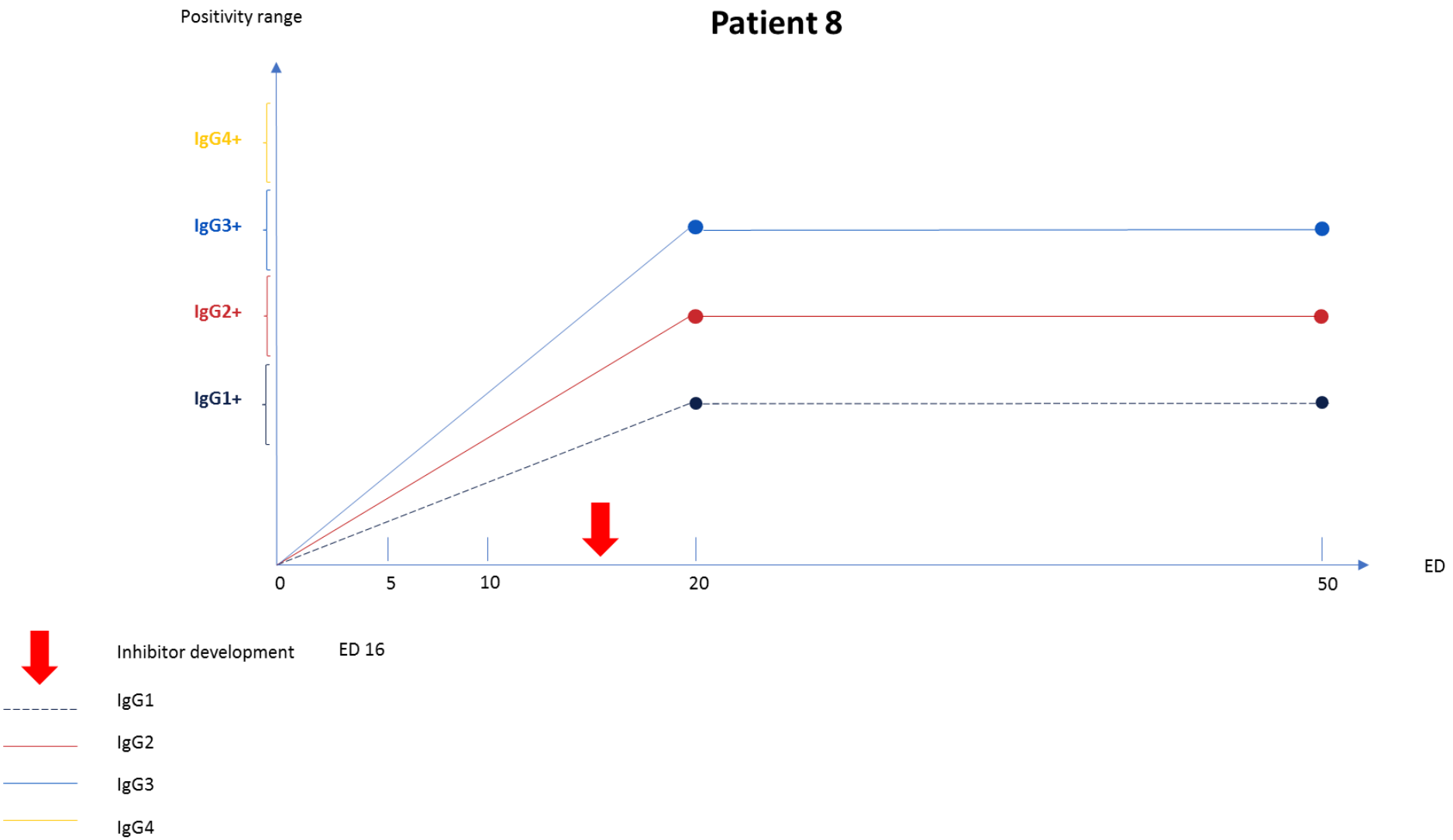


Figure 7. Schematic representation of the IgG subclass profile for the 20 patients who developed inhibitors: patient 9

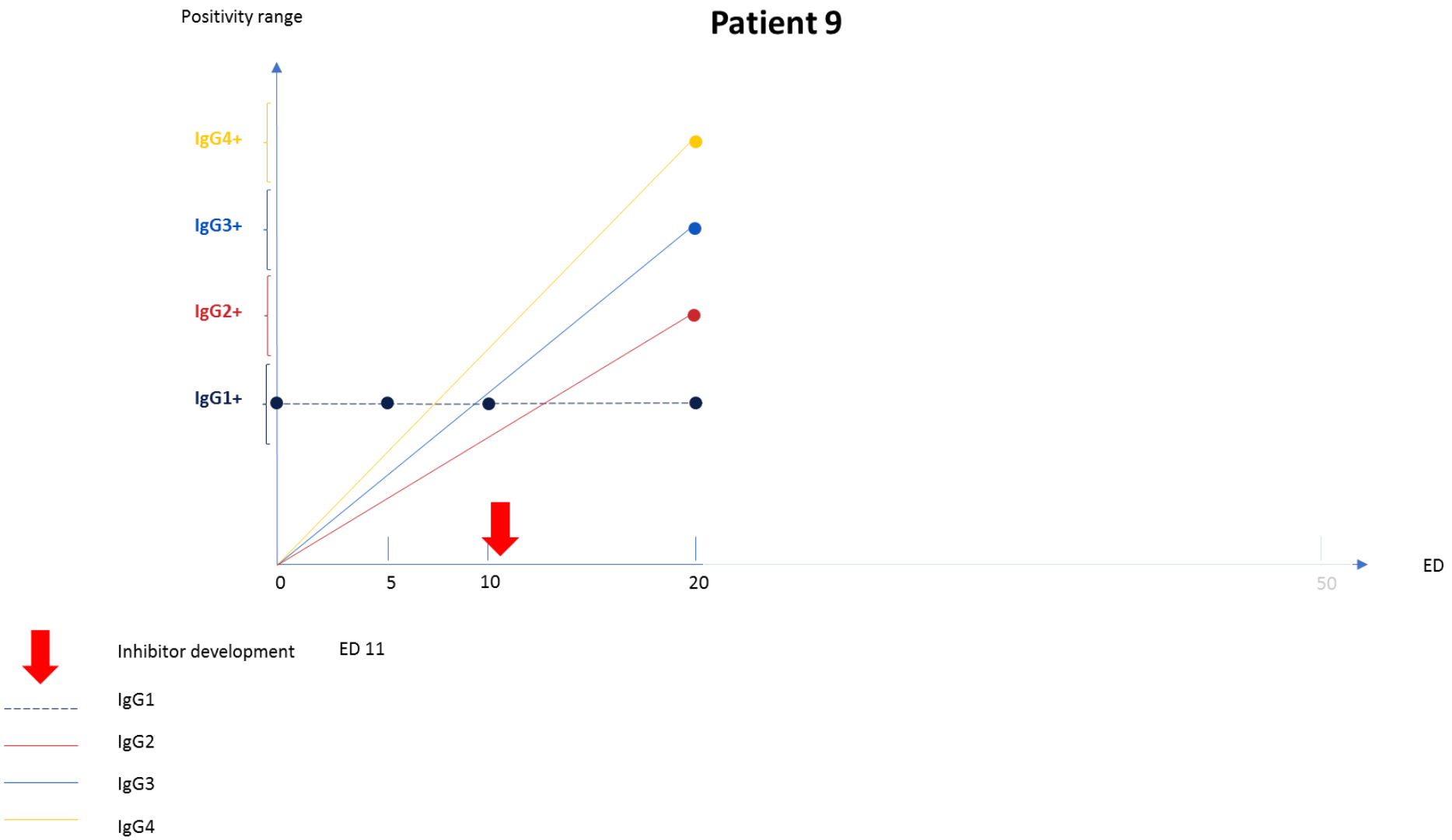


Figure 7. Schematic representation of the IgG subclass profile for the 20 patients who developed inhibitors: patient 10

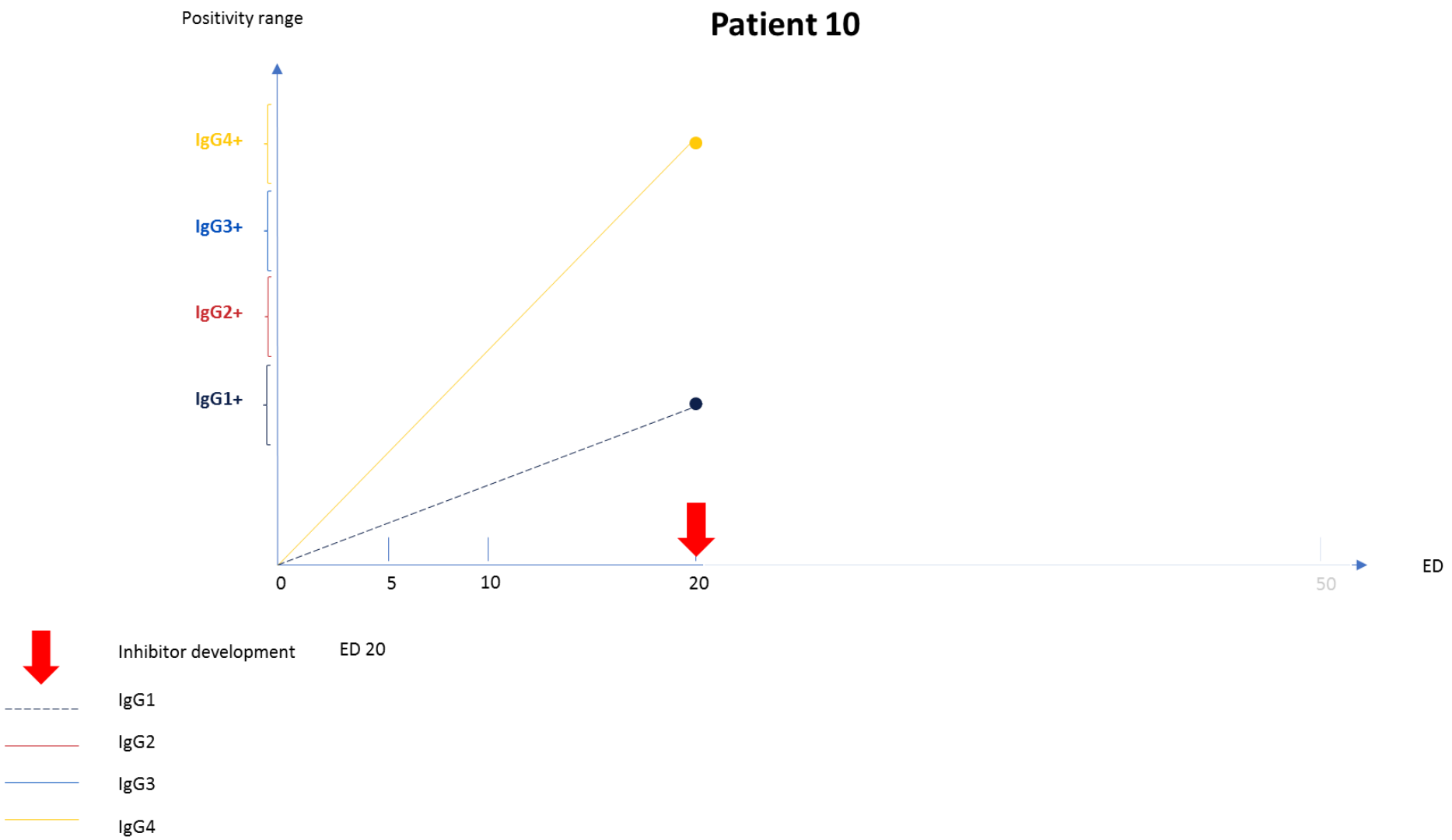


Figure 7. Schematic representation of the IgG subclass profile for the 20 patients who developed inhibitors: patient 11

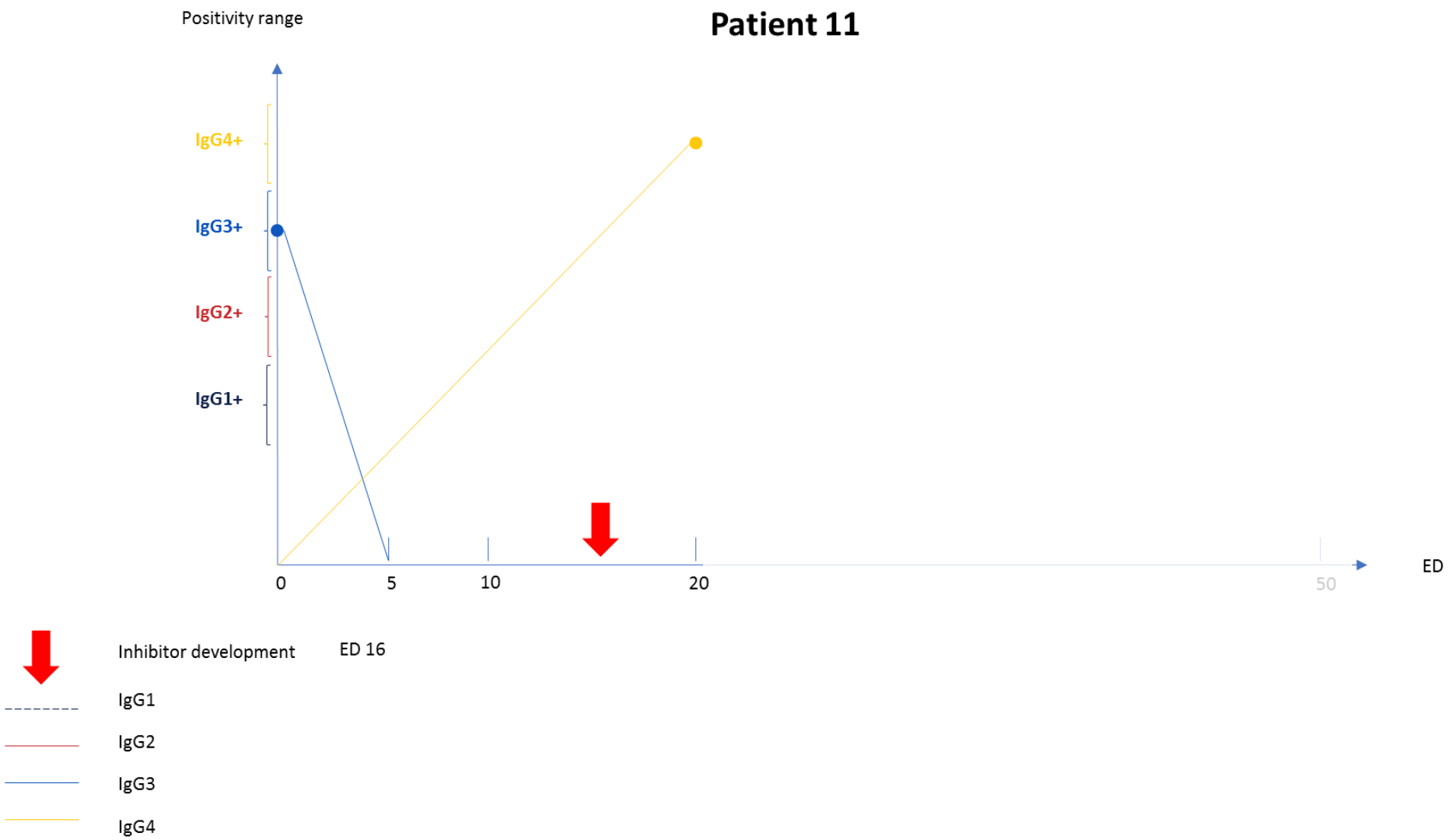


Figure 7. Schematic representation of the IgG subclass profile for the 20 patients who developed inhibitors: patient 12

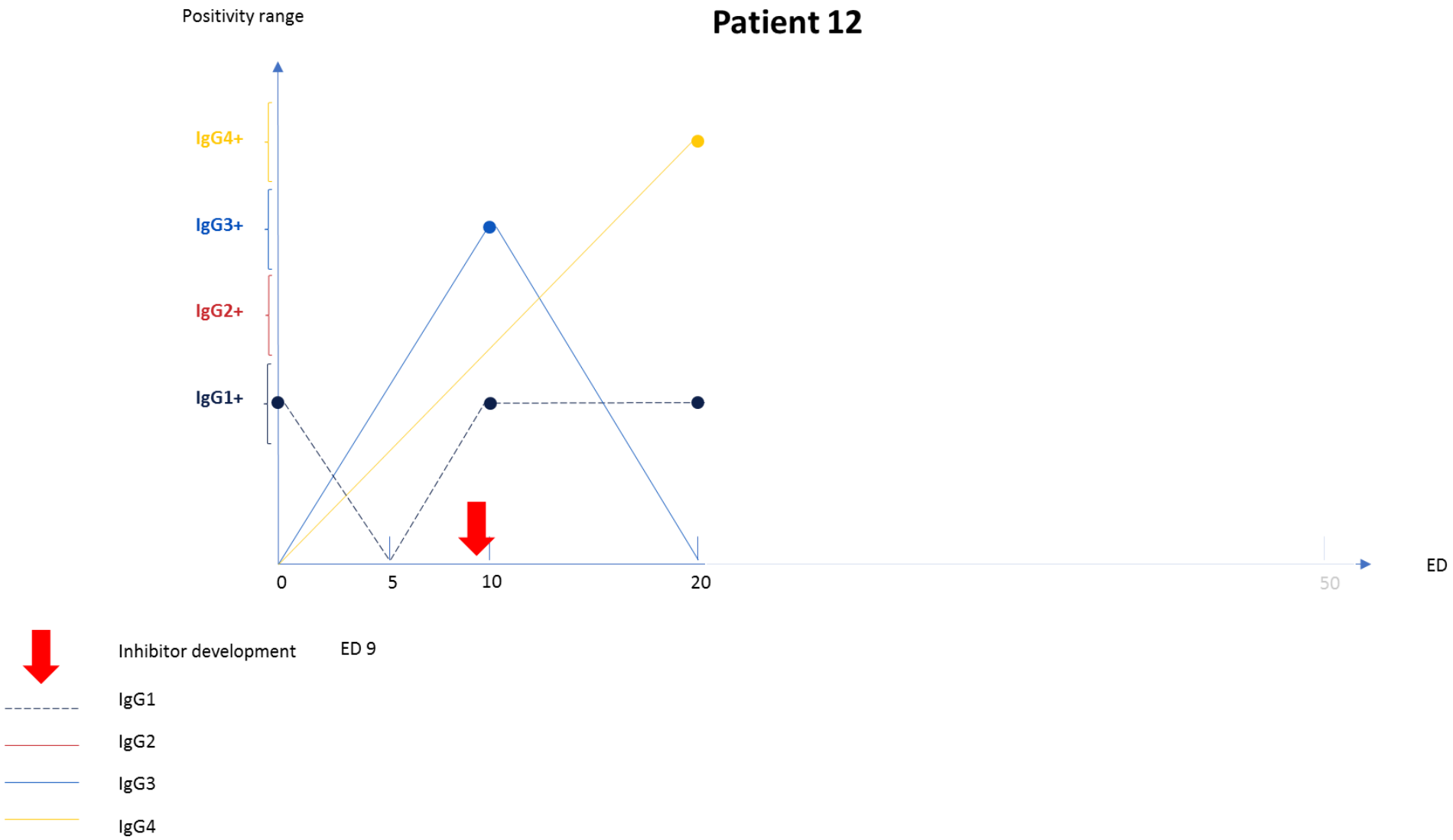


Figure 7. Schematic representation of the IgG subclass profile for the 20 patients who developed inhibitors: patient 13

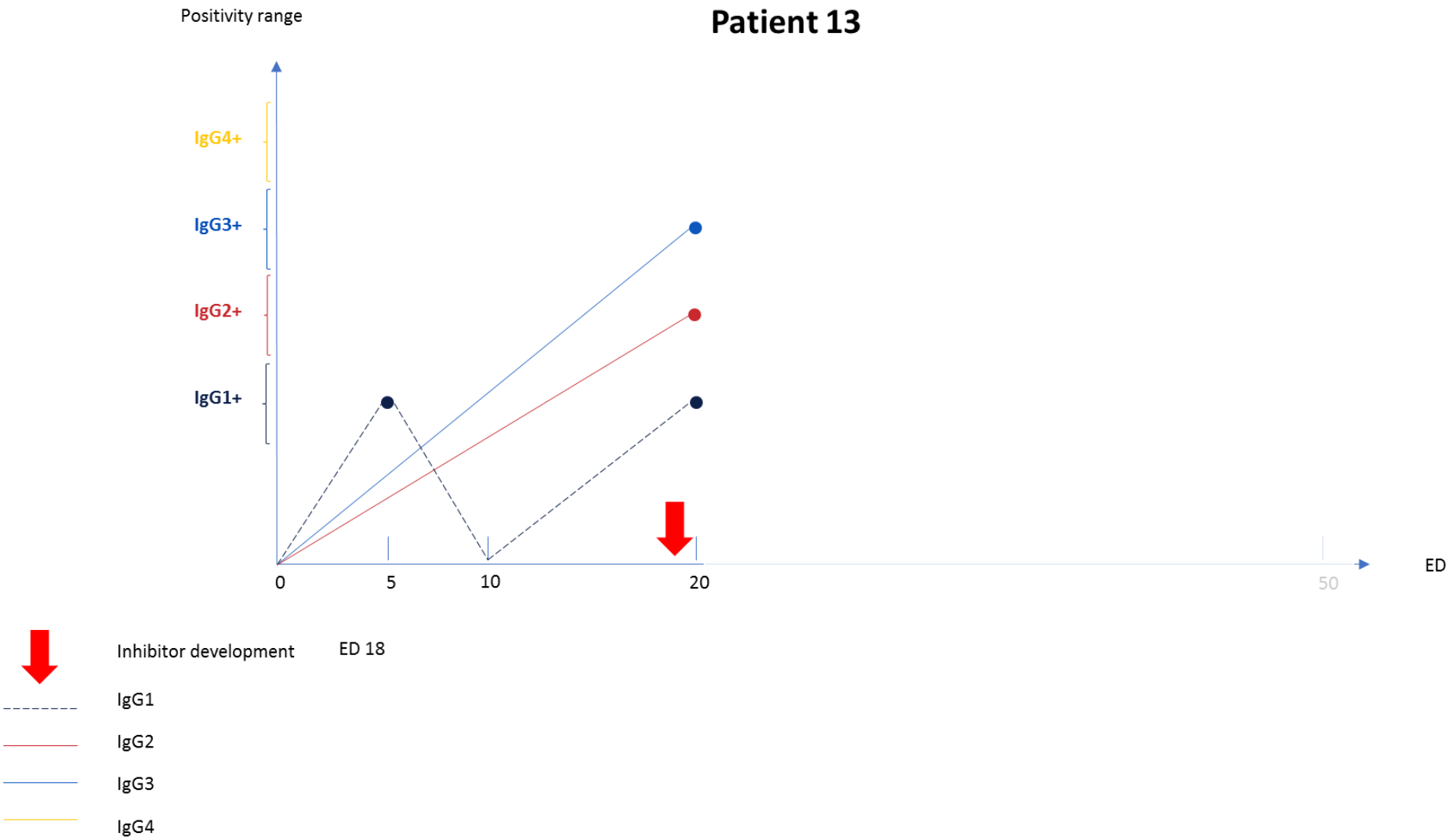


Figure 7. Schematic representation of the IgG subclass profile for the 20 patients who developed inhibitors: patient 14

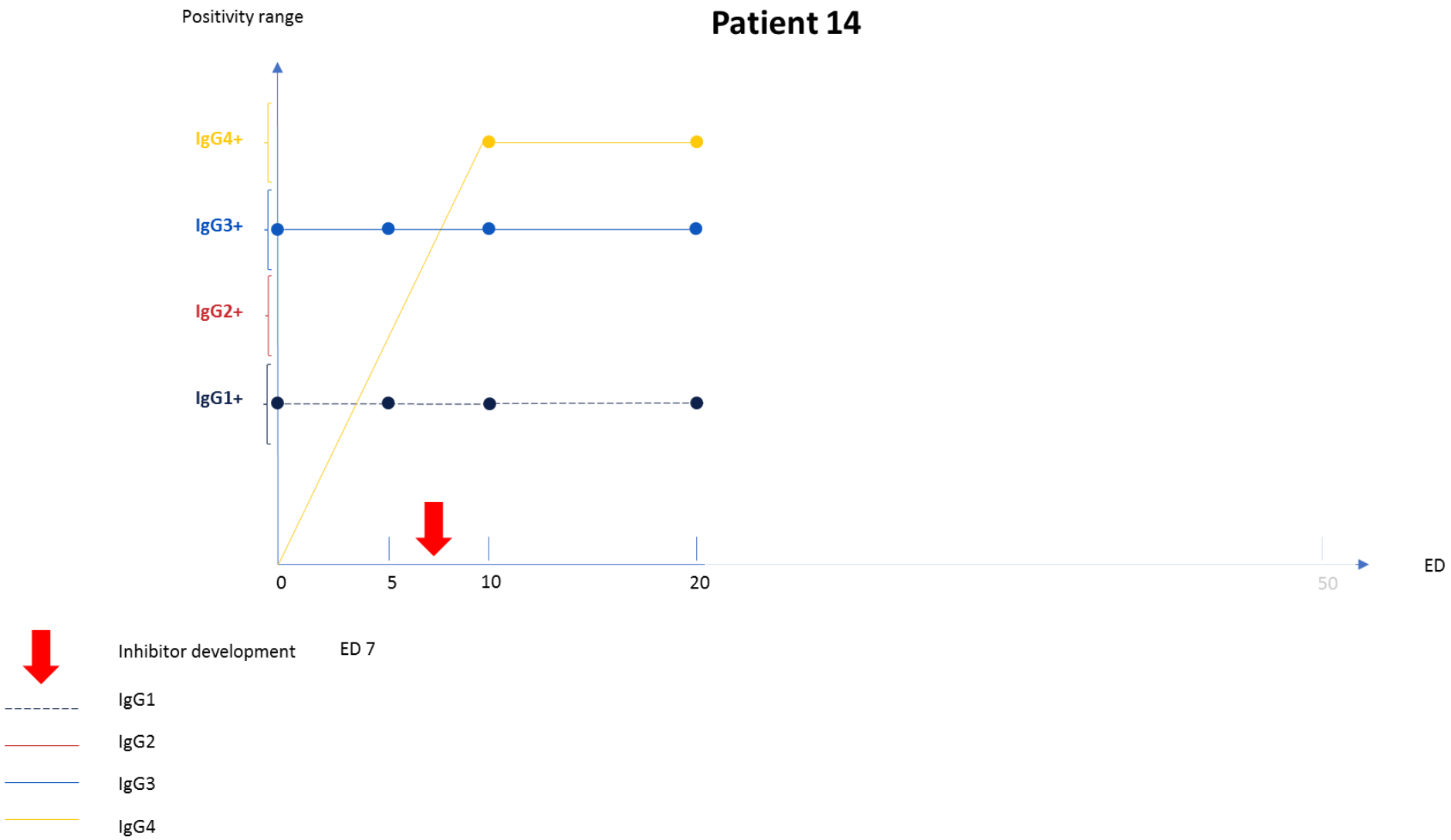


Figure 7. Schematic representation of the IgG subclass profile for the 20 patients who developed inhibitors: patient 15

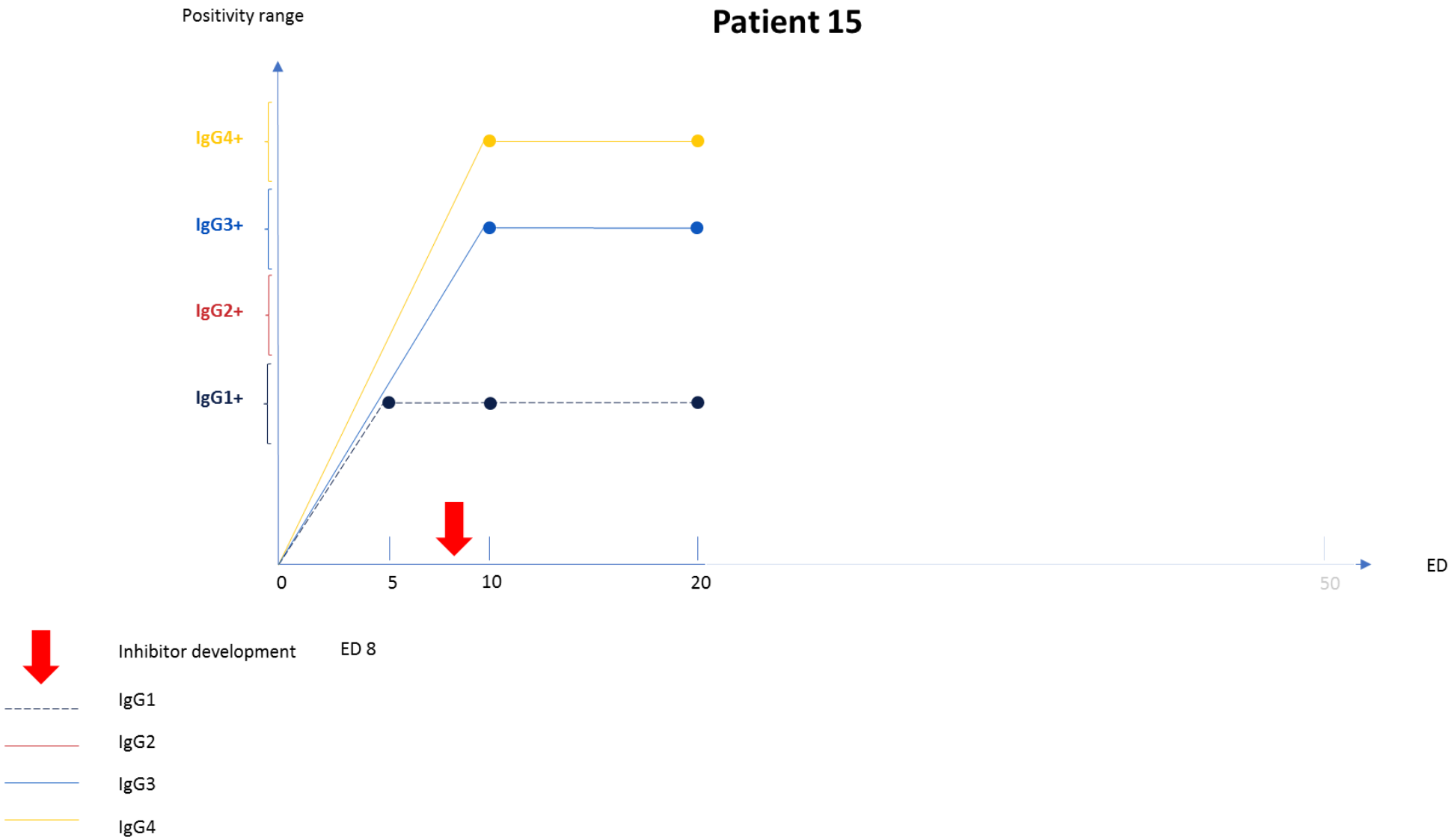


Figure 7. Schematic representation of the IgG subclass profile for the 20 patients who developed inhibitors: patient 16

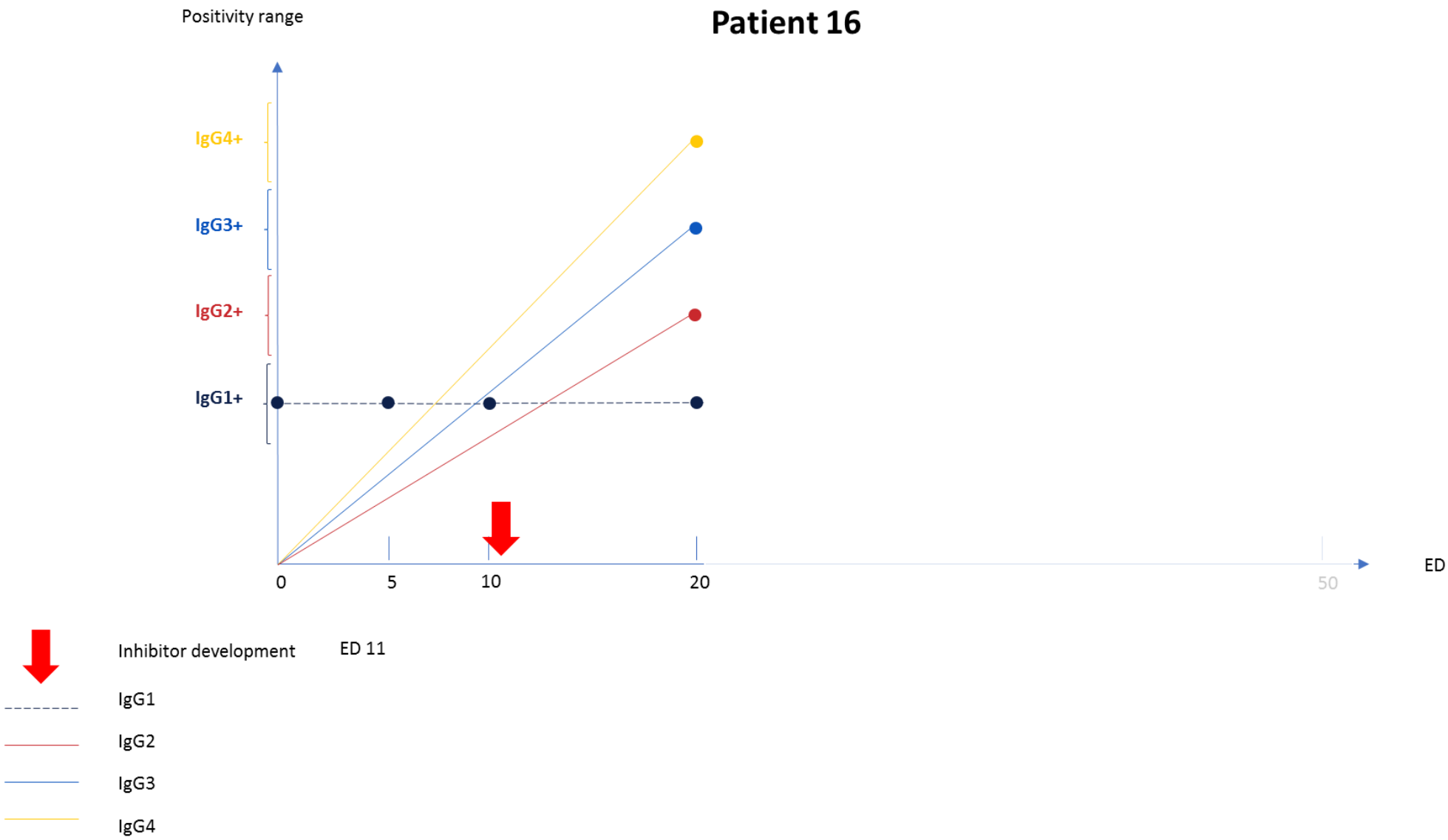
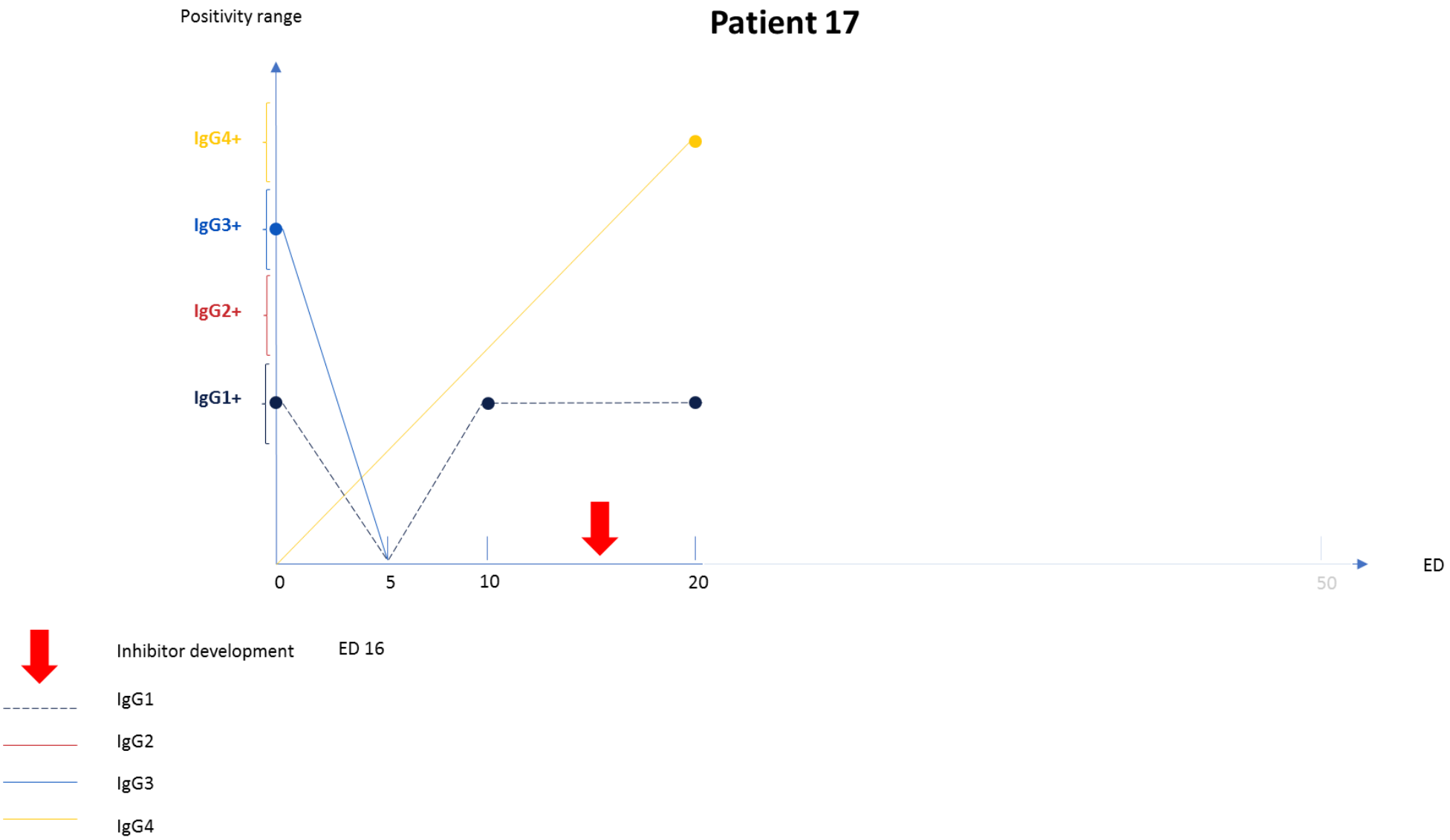


Figure 7. Schematic representation of the IgG subclass profile for the 20 patients who developed inhibitors: patient 17



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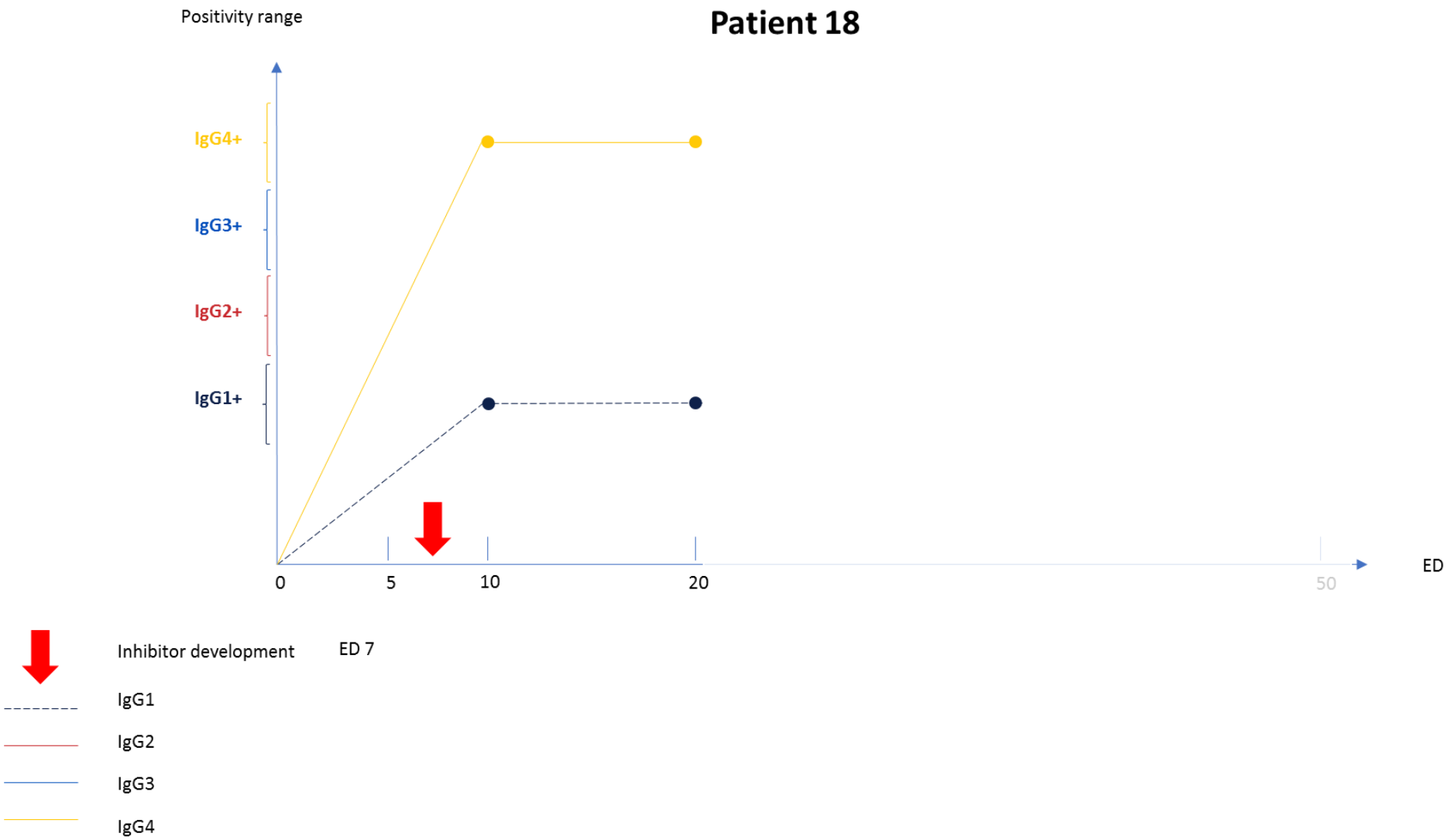


Figure 7. Schematic representation of the IgG subclass profile for the 20 patients who developed inhibitors: patient 19

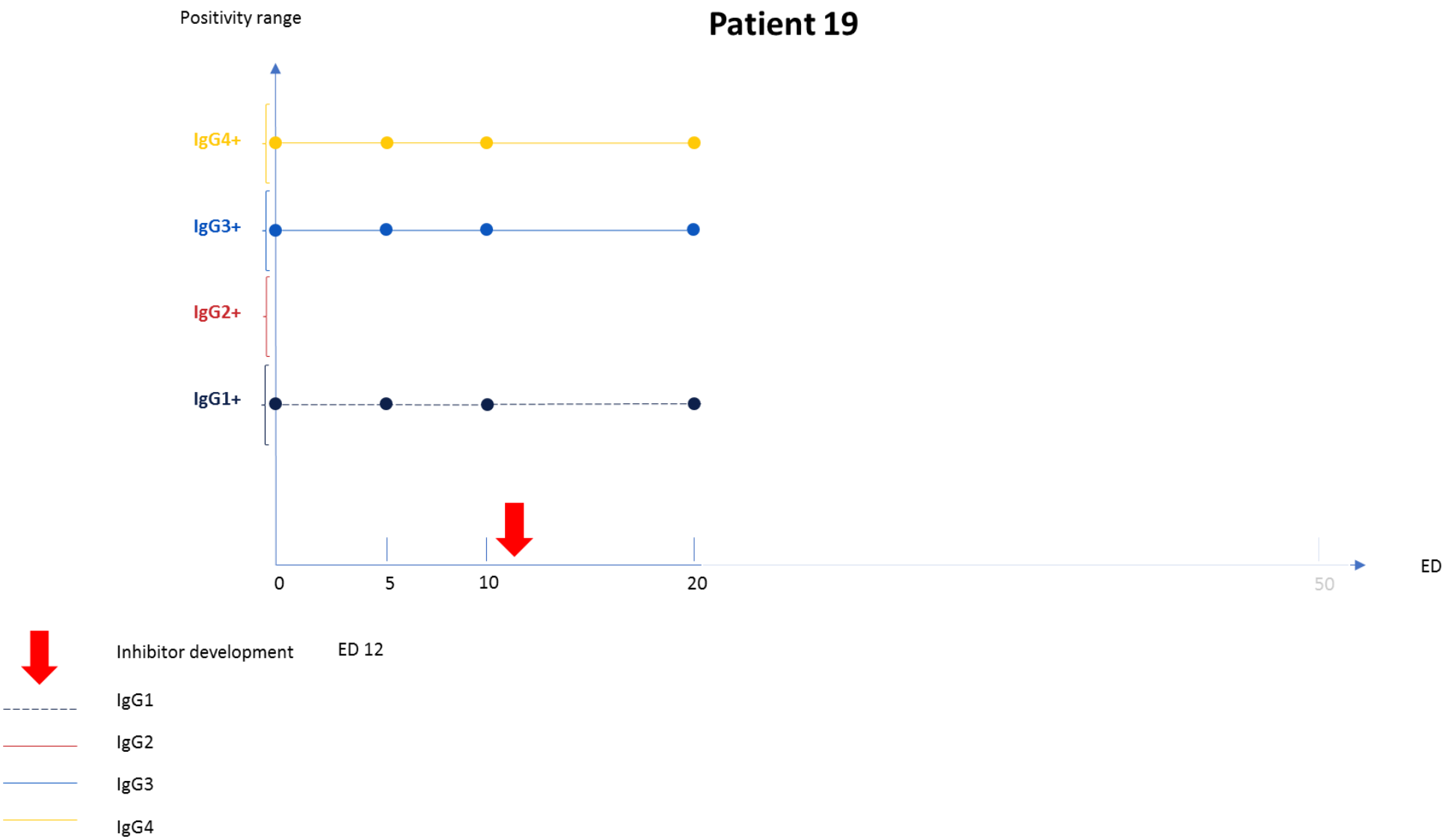
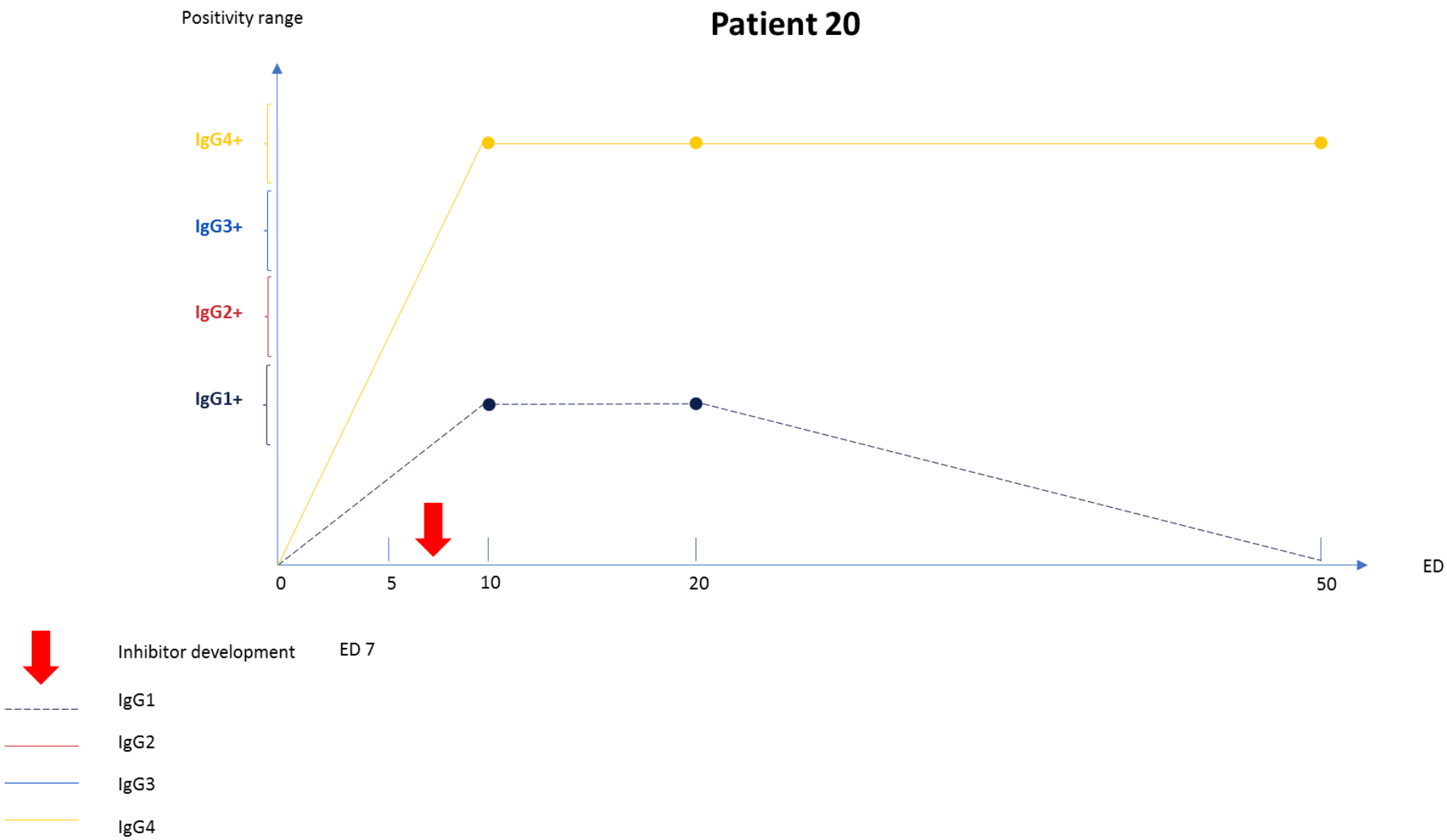


Figure 7. Schematic representation of the IgG subclass profile for the 20 patients who developed inhibitors: patient 20



Chapter IV

Table 1. Factor and non-factor therapies available in the current era for the treatment of hemophilia A with and without inhibitors.

	Description	Pros	Cons	Upcoming
Extended half-life FVIII				
• **Elocta**	Fc-fused rFVIII IV injection Approved in 2014	↑ half-life up to 19 hours	Missing long-term follow-up information on Immunogenicity	Real-world data of safety and efficacy
• **Adynovi**	PEGylated rFVIII IV injection Approved in 2015	↑ half-life up to 14.3 hours	Missing long-term follow-up information on Immunogenicity and lifelong exposure to PEG	Real-world data of safety and efficacy Approval in patients < 12 years old?
• **Jivi**	PEGylated rFVIII IV injection Approved in 2018	↑ half-life up to 19 hours	Missing long-term follow-up information on Immunogenicity and lifelong exposure to PEG	Real-world data of safety and efficacy Approval in patients < 12 years old?
• **Esperoct**	PEGylated rFVIII IV injection Approved in 2018	↑ half-life up to 18.4 hours	Missing long-term follow-up information on Immunogenicity and lifelong exposure to PEG	Real-world data of safety and efficacy Approval in patients < 12 years old?
• **BIVV001**	VWF, XTEN, Fc-fused rFVIII IV injection Approved in 2023	↑ half-life up to 40 hours	Missing long-term follow-up information on Immunogenicity	Approval in pediatric population
FVIII mimetic bispecific antibody				
• **Emicizumab**	Dimeric bispecific humanized monoclonal antibody bridging FX and FIXa	Steady state stable coagulation activity irrespective of inhibitor potency	Anti-drug antibody formation (<1% neutralizing) Thrombotic events (very rare) reported	Results on joint health of patients on emicizumab prophylaxis Approval for mild

	Subcutaneous Approved in 2018	and of age	in general but in particular in patients with inhibitors who remain vulnerable given that combination of bypassing agents (notably Feiba (APCC)) with emicizumab increases risk of thrombosis	and moderate hemophilia A
• **Mim8**	Bispecific humanized IgG4 bridging FX and FIXa Subcutaneous	*In vitro* and *in vivo*: higher hemostatic efficacy than emicizumab Lower dosage required compared to emicizumab	No results on ongoing clinical trials have been published yet	Phase 3 trial results for both adults and children with or without inhibitors as well as adults switching from emicizumab
Anticoagulent quenchers				
• **Anti-TFPI**	Humanized monoclonal antibody against the tissue factor pathway inhibitor Subcutaneous Monthly infusion	Low frequency of infusions	Thrombotic events reported in general Patients with inhibitors remain vulnerable given that combination of bypassing agents with anti-TFPI increases risk of thrombosis	Results of the adult and pediatric studies
• **Fitusiran**	Silencing mRNA of antithrombin decreasing hepatic synthesis and plasma levels Subcutaneous Monthly infusion	Low frequency of infusions	Thrombotic events Patients with inhibitors remain vulnerable given that combination of bypassing agents with Fitusiran increases risk of thrombosis	Phase 3 trial on patients without inhibitors Results of long-term follow-up in the adult study and primary results of the pediatric study
• **SerpinPC**	Serin protease inhibitor of activated protein C Subcutaneous	Phase 1 clinical results with promising safety and efficacy profiles	Thrombotic events?	Primary results of phase 2 studies on patients with and without inhibitors ≥ 12 years old

	Dosage frequency yet to be determined			Pediatric study?
Gene therapy				
• **Roctavian**	Adeno-associated virus 5 vector based gene therapy for severe hemophilia A without inhibitors and pre-existing anti-AAV5 antibodies Conditional EMA approval in 2022 FDA yet has to express opinion	Transformation of severe to moderate-mild bleeding phenotype in responders Only potential possibility of cure	Not approved for patients with pre-existing anti-AAV5 antibodies Initial immunosuppression required Decrease of FVIII activity with time, requiring a subset of patients to reintroduce replacement therapy Transmanitis and potential long-term liver damage Long-term surveillance required for unspecific integrations and oncogenic events Costs and access	Long-term safety and efficacy surveillance data expected Primary results of trial on patient with inhibitors, present or history Trial on children? Reevaluation of costs and reimbursability

Appendices

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