

Immune tolerance induction in hemophilia

The development of inhibitors is the main complication of hemophilia therapy. Inhibitors occur in 25–30% and in 2–5% of patients with severe hemophilia A and B, respectively. They render treatment and prevention of bleeds difficult. The only known therapeutic strategy able to eliminate inhibitors is immune tolerance induction (ITI) that consists in regular high-dose FVIII/FIX infusions. ITI is a demanding treatment both for patients/parents and clinicians, it is costly, but provides 60–80% chance of success. Although used since late 1970, many aspects of ITI still needs to be optimized and further investigated. This review is aimed at reporting what is known from the published literature and what still need to be investigated in this field.

Keywords: hemophilia A • hemophilia B • immune tolerance induction
• immunosuppressive drugs • inhibitors • rituximab

Hemophilia is a rare inherited bleeding disorder due to the deficiency of factor VIII (FVIII, hemophilia A) or factor IX (FIX, hemophilia B) in plasma [1]. Replacement therapy is the cornerstone of hemophilia management since it allows to control active bleeding by on demand episodic treatment and/or to prevent recurrent bleeds by regular prophylaxis [1]. The development of neutralizing alloantibodies directed against FVIII or FIX (referred to as inhibitors) is the main complication of hemophilia treatment because it renders bleeding control difficult and standard prophylaxis unfeasible [1]. Inhibitor development is more common among patients with hemophilia A than in those with hemophilia B, and in patients with severe FVIII/FIX (below 1%) than in those with moderate/mild hemophilia [1]. The risk of developing inhibitors is maximum after the first 10–15 exposure days to the antigen (i.e., FVIII or FIX concentrates), hence inhibitors occur mostly in children with severe hemophilia A [2]. Among these inhibitors almost one-third are transient and spontaneously disappear without sequelae and the need for specific treatment regimens.

On the other hand, in the presence of persistent high-titer inhibitors, standard FVIII replacement therapy is no longer effective and recurrent joint bleeds are commonly managed by on demand treatment with bypassing agents (i.e., recombinant activated factor VII, rFVIIa, and activated prothrombinic complex concentrate, aPCC) whose hemostatic efficacy may be suboptimal as compared with FVIII replacement therapy. This produces a relevant morbidity and chronic degenerative joint damage that still characterize the natural history of the disease in inhibitor patients [3]. In this light, in order to halt disease progression and maintain a healthy joint status, the attempt at eradicating inhibitors is mandatory especially in young children. Up to now the unique strategy that has been proven to be able to eradicate inhibitors is immune tolerance induction (ITI) treatment, first reported in late 1970s in Germany [4]. This treatment generally consists in the regular administration of FVIII or FIX in order to render the immune system tolerant to the antigen by preventing further production of the antibodies. It is a demanding therapeutic strategy since it

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implies frequent intravenous injections (i.e., daily or every other day) in subjects with poor venous accesses as children and for a rather prolonged time (i.e., in median 12 months) [5]. Nonetheless, despite these drawbacks, ITI is successful in up to 60–80% of cases [6], although many patient- and treatment-related variables may influence the final outcome. Most of these variables have been identified while other still need to be further investigated [5].

ITI optimization is a priority for hemophilia treaters and the identification of predictors of response is important in order to offer ITI to patients who may benefit the most from it and to tailor it in a proper way, thus avoiding a waste of resources.

Finally, ITI has a relevant economic burden that is mostly related to the cost of factor concentrates used at high doses for prolonged time, but also to the cost of other medical procedures related to ITI feasibility. These include central venous lines insertion, management of catheter-related complications (i.e., infections, thrombosis) and treatment of breakthrough bleeds with by-passing agents during the first phase of ITI. The aims of this review are:

- To evaluate what is the current knowledge on ITI treatment;
- To highlight which are the gray areas that still need to be further investigated in this field.

ITI & hemophilia A: data from clinical observation

All the data on the practice of ITI in hemophilia A come from national and international registries [7–13], several observational retrospective and prospective studies [14–37], and one randomized clinical trial [38]. The analysis of published data allowed to identify several predictors of success or failure of ITI that are currently used to guide decision-making on eligibility and feasibility of ITI in inhibitor patients.

Definition of ITI outcome

Success rates of ITI in hemophilia A have been defined by stringent clinical and laboratory features [39] and definition of success was first established by international consensus (Consensus Proceedings from the Second International Conference on Immune Tolerance Therapy held in Bonn in 1997, unpublished) as follows:

- Success – undetectable inhibitor titer (<0.6 BU/ml), FVIII *in vivo* recovery $\geq 66\%$ of the expected value, FVIII half-life ≥ 6 h after a 72-h wash-out period from last infusion and the absence of anamnestic increase of inhibitor titer upon further FVIII exposure.

Afterward, an international expert panel drew Consensus Recommendations on ITI [5] agreeing upon the aforementioned definition of success and further defining partial success and failure as follows:

- Partial success – a reduction of the inhibitor titer to <5 BU/ml, FVIII recovery <66% of the expected value and FVIII half-life <6 h after a 72 h wash-out period associated with clinical response to FVIII replacement and no increase of the inhibitor titer above 5 BU/ml over a 6 month period of on demand treatment or 12 months of prophylaxis [5].
- Failure – inhibitor titer decline less than 20% over any 6-month period after the first 3 months of ITI or failure to achieve success or partial response after 33 months of ITI.

The same definitions for success and failure were adopted to design the International ITI Study (I-ITI Study) [38], where on the contrary partial response was defined in a different way as follows:

- Partial response – undetectable inhibitor titer but persistently abnormal FVIII recovery or half-life after 33 months of ITI in association with a clinical response to FVIII replacement therapy without an anamnestic increase of the inhibitor titer [38].

In addition, inhibitor relapse was defined as follows:

- Relapse – inhibitor recurrence during the 12 month follow-up period on prophylaxis after success as evidenced by recurrent positive inhibitor titer or impaired FVIII pharmacokinetics (PK) [38].

Patient-related predictors of success

Among patient-related characteristics that might be associated with ITI outcome age at ITI start, race/ethnicity, FVIII genotype and age at inhibitor development have been considered so far.

Age at ITI start

Published data are quite controversial with this respect: in the International ITI Registry (IITR) [10] higher success rates were observed in subject treated within 20 years of age, while in the Spanish Registry [9] higher success rates were observed in patients who started ITI at an age older than 7 years. On the contrary, in a recent case series no correlation between age at ITI start and ITI outcome was found [36]. These differences may be due to the fact that the time elapsing between inhibitor development and ITI start rather than age *per se* influences most ITI outcome considering that the longer inhibitors persist the more difficult is to revert the immune response abolishing antibodies production. In this light adult

age *per se* should not be considered as a predictor of ITI failure.

Race/ethnicity

In the North American Immune Tolerance Registry (NAITR) [11] no difference in success rates was found between Africans, Hispanic/Latinos and patients of other races, while a retrospective single-center analysis suggested a significantly lower ITI success rate among African Americans (58 vs 92% in Caucasians) [31]. This difference might be due to significantly higher pre-ITI inhibitor titers (see following paragraph) in the African American group [31]. Race and ethnicity were analysed also in the I-ITI Study [38] and they had no impact on ITI outcome, although it should be underlined that African Americans represented only a minority of enrolled patients in that study (i.e., 8%) [38].

FVIII genotype

The impact of FVIII genotype was reported in two observational studies [16,18], but first purposely investigated in the frame of the Italian PROFIT Registry [12]. There a higher success rate was observed in patients bearing FVIII gene mutations predictive of a low inhibitor risk as compared with those with high-risk mutations (81 vs 47%; adjusted odds ratio 6.2, 95% CI 1.1–36.0) [12]. Similar findings have been reported in other two series in which the presence of large deletions of FVIII gene was associated with ITI failure [40,41]. Indeed, the identification of such pretreatment predictors would facilitate clinicians in drawing a risk profile for each inhibitor patient potentially candidate to ITI. However, such a role needs to be investigated in the frame of large cohort studies in which all potential confounders and risk factors are taken into account.

Age at inhibitor development

An age at inhibitor development below 2.5 years was associated with earlier achievement of success only in one study [14], but not further confirmed and/or investigated as independent predictor of success in following studies.

Treatment-related predictors of success

Inhibitor titers at various time points prior and during ITI have been confirmed as independent predictors of ITI outcome. Their role have been explored and consistently confirmed across different publications even if with some differences related to the different nature of the cohorts/series analysed. Less robust and/or conclusive data have been produced with respect to time elapsing between inhibitor development and ITI start, ITI interruptions, FVIII product type, FVIII dose and treatment schedule.

Pre-ITI inhibitor titer

A low inhibitor titer at ITI onset (i.e., below 5 or 10 BU/ml across published data) is currently recognized as one of the main determinant of ITI success as reported in several retrospective and prospective studies [7–12,16–22,25,28,31,32,36].

Historical inhibitor peak

The inhibitor peak ever reached prior to ITI start distinguishes inhibitors in high- (≥ 5 BU/ml) or low-responding (always < 5 BU/ml) and can be considered as a marker of the intensity of the immune response against FVIII. Several studies showed that high historical peaks were invariably associated with ITI failure. However different cut-offs were used to categorize these historical peaks and peaks exceeding 10 [32], 20 [38], 40 [14], 50 [12,25,28,31], 100 [16,17,19,20] or 200 [7,8,10,11,13,36,42] BU/ml were variably taken into account accordingly to the different study population considered in each study. In fact, for instance, in the I-ITI Study only patients with a good risk profile (see below) with a historical peak < 200 BU/ml were included, resulting in a median historical peak of 22 BU/ml that was used as cut-off value for all analyses [38]. By univariate analysis the higher was the peak titer the higher was the risk of failure, but this was not confirmed in the multivariate model indicating that in good risk patients this variable has less impact on ITI outcome [38].

Inhibitor peak on ITI

Similarly to historical peaks, also inhibitor peaks achieved during ITI were inversely associated with ITI success rates in some studies [12,21,38]. In particular, in the Italian PROFIT Registry an ITI peak titer below 100 BU/ml was associated with higher chance of achieving tolerance [12] and in the good risk population of the I-ITI randomized Study this variable resulted the only independent predictor of success in the multivariate model [38].

Time period elapsing between inhibitor development & ITI start

Data from IITR and NAITR showed that the shorter was the time elapsed between inhibitor development and ITI start, the higher was the chance of achieving tolerance with a cut-off settled at 5 years [7,8,10,11]. Similar data were confirmed in subsequent studies where a period of 2 years between inhibitor development and ITI start was associated with the highest success rates [20,25,36]; on this basis ITI onset within 2 years from inhibitor development was considered as an inclusion criteria for the I-ITI study in which only good risk patients were enrolled [38].

ITI interruptions

Data from the German experience showed that ITI interruptions for more than 2 weeks were associated with lower success rates and with longer duration of ITI courses [16,42,43].

FVIII product type

Published data support the evidence that tolerance can be achieved at high rates ranging between 60 and 80% by using either plasma-derived [18–20,23–25,27–30,34,36,37] or recombinant [15,18,19,21,22,26,28,31,32,44] FVIII concentrates. Data from Registries [9–13] and from the I-ITI randomized study [38] are not informative with this respect since the distribution of products used is skewed in the former and the vast majority of patients (i.e., 90%) enrolled in the I-ITI study received recombinant products. So far no robust evidence support the superiority/inferiority of certain types of FVIII concentrates to be used for ITI and the role of product type as well as of von Willebrand factor (VWF) content as predictor of ITI outcome is still matter of debate (see below).

FVIII treatment schedule

Also the role of FVIII doses and dosing regimens as predictors of ITI success/failure has been greatly debated owing to the controversial data coming from the registries. In fact in the IITR higher success rates were associated with the use of doses ≥ 200 IU/kg/day [10], while in the NAITR and in the Spanish Registry similar figures were yielded with FVIII doses < 200 IU/kg/day [9,11]. Moreover, the NAITR reported a more significant impact of doses on time to achieve tolerance than on success rate *per se* [7,8,11]. A meta-analysis of data coming from both registries ultimately demonstrated that in patients with a good risk profile (see below) FVIII dose did not impact on success rates [7]. Indeed similar success rates have been obtained by using a very wide dose regimens ranging from low-dose protocols with 25 IU/kg FVIII every other day as in the Van Creveld model [45] to the epitome of high-dose protocols represented by the Bonn protocol with 150 IU/kg FVIII twice daily [43]. Between these two extremes, a varied combination of doses and dosing intervals were described across literature with no significant differences on success rates. In fact, the most common dose used in the field practice is 100 IU/kg every day or every other day as reported in the NAITR (52% of cases) [7,11], in the IITR (48% of cases) [10], in the Italian PROFIT registry [12] and in other observational studies [18,19,21,22,24,26,27,30,32,34,36,44,46,47].

Due to the wide variability of dosing regimens used for ITI, the need for understanding if they were all comparable not only in terms of success rates but also with

respect to cost-effectiveness and morbidity (i.e., bleeding frequency during ITI) was the main reason for designing the first international randomized trial for inhibitor patients undergoing ITI [38]. The I-ITI study was aimed at comparing a low-dose (i.e., 50 IU/kg thrice weekly) versus a high-dose (i.e., 200 IU/kg/day) treatment arm in patients with good risk predictors of response [38]. As expected the success rate was rather high and similar in the two arms (70% for both); however, patients treated with the high dose reached the major endpoints (namely, undetectable inhibitor titer and normal FVIII PK parameters) in a significant shorter time period than those treated with the low-dose regimen [38]. Moreover, the study showed an unanticipated significant impact of dose on ITI-related morbidity since patients included in the low-dose arm bled significantly more than those in the high-dose arm, especially in the first period of ITI when inhibitor titer was still detectable [38]. The great impact of this safety result led to a premature study closure.

With respect to ITI dosing regimen one peculiar and unique ITI schedule is represented by the Malmö protocol developed in Sweden in the 1980s by Prof. Nilsson after the clinical observation of a patient with hemophilia B with inhibitors [48]. Since then it has been used both in hemophilia A and B [49,50]. According to this protocol FVIII is given every 8–12 h and it is associated with extracorporeal immunoadsorption and immunosuppressive drugs from the beginning in order to obtain a rapid drop of the inhibitor titer. This allows to maintain FVIII levels above 30 IU/dl during the first days. Afterward, as soon as anamnestic response occurs, the intervals of FVIII administrations are shortened at 6 h increasing the total daily dosage. FVIII treatment is maintained until inhibitor disappearance and then tailed off to regular prophylaxis [50]. This demanding strategy has been applied in the past in hemophilia A patients with a success rate of 67% [51], but not further widespread due to the fact that results obtained were not superior to ITI courses performed with FVIII concentrate only. Its use can be considered in particular cases in which inhibitor titer reduction is urgent to ensure hemostasis by restoring a normal response to FVIII replacement (i.e., life-threatening bleeds, major surgery).

Prognostic profile of patients undergoing ITI

The issue around ITI prognostic profile is clinically relevant taking into consideration that ITI management, particularly the choice of type of FVIII concentrate, dosing and duration, is usually influenced by the patient's risk profile. Based on literature reviews and data from registries [5–7], patients with a high probability of achieving success ('good risk') are those with

a pre-ITI titer <10 BU/ml and a historical peak titer <200 BU/ml. In contrast, patients with a low probability of inhibitors eradication ('bad risk') are those with a pre-ITI titer \geq 10 BU/ml or a historical peak titer \geq 200 BU/ml.

The same definitions were proposed by UK Haemophilia Centre Doctors Organization in the frame of the recently published national guidelines on the management of inhibitor patients [52].

A more stringent combination of criteria was adopted to select 'good risk' patients for inclusion into the I-ITI Study [38] as follows:

- Age <8 years at time of study entry;
- Historical peak titer \leq 200 BU/ml;
- Decrease of the inhibitor titer to \leq 10 BU/ml in <24 months;
- Pre-ITI titer \leq 10 BU/ml;
- Patients naïve to previous ITI courses.

On the other hand, in several studies it was considered sufficient to have at least one negative predictor to label patients as 'bad risk' with a poor chance of ITI success [16,20,23,30,33,36,37,53].

ITI & hemophilia A: what still needs to be investigated

Immunological mechanisms are potential novel therapies for immune tolerance. The mechanism by which immune tolerance is induced toward inhibitors is a complex mechanism, not yet fully understood. Anti-FVIII immune response takes place into two phases. In a first phase, the antigen (i.e., FVIII/FIX) will be endocytosed, processed and presented to FVIII specific CD4⁺ T cells by antigen-presenting cells (APCs). In a second phase, additional interactions will occur between CD4⁺ T cells and FVIII-specific B cells in order to permit B cell activation, cellular differentiation into plasma cells and antibody secretion by FVIII-specific plasma cells. Indeed, inhibitor development is depending of both phases whereas the inhibition of interactions between APCs and T cells or T cells and B cells is believed to be enough to restore antigen tolerance. Tolerance can be defined as a state of unresponsiveness to an antigen by an immune system which is fully competent. It can be induced by three basic mechanisms: ignorance, anergy and deletion [54–56]. All these mechanisms may involve both B- and T-cell compartments that are strictly interrelated in the modulation of the immune response. Ignorance exists when the interaction between the antigen (i.e., FVIII/FIX) and immunoglobulins and/or T-cell receptors is absent,

as for instance in the presence of mutated major B- and/or T-cell epitopes in the antigen molecule. Anergy is a mechanism in which the lymphocyte is intrinsically functionally inactivated following an antigen encounter, but remains alive for an extended period of time in a hyporesponsive status [54–57]. This could be induced in B cells by cross-linking surface immunoglobulins as anti-idiotypic antibodies and in T cells by neutralizing CD40-CD40 ligand interactions or by blocking the signals generated on the surface of APCs [58,59]. In fact, *in vitro* studies showed that anti-idiotypic antibodies are present in plasma from patients who underwent successful ITI but not in plasma from those who failed or had a partial success [57,60], and that the neutralizing activity of these antibodies increases over time during successful ITI shifting the immune system to a state in which alloimmunity is prevented [57,60]. Deletion is due to cell death and can be obtained by specific hyperstimulation of both B and T cells that leads to the hyperexpression of a surface molecule named Fas whose activation by Fas ligand results in cell death [54]. All in all, by a clinical perspective, if one considers the conventional high-dose daily treatment with FVIII for ITI, this treatment is more likely to result in cell deletion occurring in the periphery, whereas low-dose protocols would be more prone to trigger anergy by CTLA-4 stimulation at T-cell level or by cross-linking of B-cell surface immunoglobulins.

At the start of ITI, the immune system of inhibitor patients is probably characterised by the presence of three different classes of cells: FVIII-specific memory CD4⁺ T cells and B-cells, and anti-FVIII antibody-producing plasma cells. It is assumable that these cell subsets need to be inactivated or eliminated during the course of a successful ITI; however, only memory T and B cells express FVIII-specific receptors at variance with plasma cells. Therefore, direct antigen-specific inactivation or depletion of plasma cells by ITI seems rather unlikely. However, the inactivation/depletion of memory B- and T-cells may result in an impoverishment of the pool of long-lived plasma cells and the maintenance of FVIII-specific tolerance. It has been shown that FVIII-specific memory B cells are rarely detected in peripheral blood samples from healthy individuals as well as in hemophilic patients without inhibitors, that their amount is widely variable in patients with inhibitors depending on antigenic challenging and that they are no longer detectable in patients who achieved tolerance [61]. *In vivo* experiments in large cohorts of patients undergoing ITI confirming such hypotheses are still lacking and limited data pertain to animal models [62].

Other mechanisms to induce tolerance have been investigated in animal models. The administration of

viral vectors carrying the human FVIII gene in mice with anti-human FVIII inhibitors resulted in the disappearance of the antibodies suggesting that tolerance may be induced by sustained expression of the antigen [63]. The administration of purified FVIII C2 domain via mucosal route (i.e., nasal and oral) induced tolerance in hemophilic mice [64] and inhibitors were eradicated in hemophilic dogs treated with liver-directed gene therapy by adeno-associated viral vectors [65].

All this experimental hypotheses need to be evaluated *in vivo* in the clinical setting of patients with inhibitors undergoing ITI. However, some data have been obtained in a longitudinal analysis of IgG subclasses of anti-FVIII antibodies during the course of ITI in 14 patients [66]. Those data revealed that in low-titre inhibitor patients antibodies consisted primarily of IgG1 whereas IgG4 were predominant in patients with high-titre inhibitors who needed prolonged ITI treatment or who failed [66]. In accordance with this observation, in a larger patient population it has been reported that IgG1 and IgG4 are the most abundant subclasses in inhibitor patients and that IgG4 are completely absent in patients who do not develop inhibitors as well as in healthy individuals [67]. In fact, differences in IgG subclasses are indicative of different T-helper populations that regulate the differentiation of B cells in antibody-producing cells. Further studies are needed to address whether monitoring IgG subclasses during ITI can be of help in the early identification of patients at high risk of ITI failure.

The role of VWF in ITI

The issue concerning the role of VWF (contained in many plasma-derived FVIII concentrates) in the promotion of immune tolerance toward FVIII is still controversial. In normal plasma FVIII is bound non-covalently to VWF mainly through interaction with the light chain. VWF prolongs FVIII half-life and protects it from proteolysis. In patients with hemophilia exogenous FVIII forms a complex with endogenous VWF very rapidly. Considering the importance of VWF for functional integrity and survival of FVIII in plasma, it is easy to speculate that VWF contained in some concentrates may have a role in tolerance induction by providing specific protection against FVIII-inhibitor interactions that often occur at the same binding site on the light chain. In fact, it has been shown *in vitro* that VWF competes with inhibitory antibodies at some binding sites on FVIII C2 domain [68,69]; however, there is very limited evidence to prove that this is the case *in vivo*. In a small case series it was shown that the most common inhibitor epitopes were located in the FVIII C2 (light chain) and A2 (heavy chain) regions and that the pre-existence or emergence of the A2

epitope specificity was associated with higher chance of ITI failure even if FVIII/VWF-containing products were used [46]. In Germany lower ITI success rates were achieved by using recombinant than VWF-containing products (29 vs 91%, respectively) [70] suggesting a role for VWF content of FVIII products in favouring tolerance. Nevertheless, in that experience the duration of ITI courses performed with recombinant and VWF-containing products was significantly different [70]. On the other hand, the use of VWF-containing products for ITI in poor risk patients was associated with rather high success rates suggesting a role for this product type in this subgroup of patients [23,24,27,30,34,36,46].

The role for FVIII product type as predictor of ITI outcome is currently prospectively investigated in:

- The ongoing Italian registry [12];
- A multinational observational ITI Study (i.e., the ObsITI study) [71];
- A randomized controlled trial comparing the use of VWF-containing products versus recombinant FVIII for high-dose (i.e., 200 IU/kg/day) ITI treatment in naïve poor risk; patients (i.e., the RESIST study) [53].

The role of immunomodulating &/or immunosuppressive drugs

Despite considerably high success rates, there is a 20–30% of patients who still fails to achieve tolerance after ITI. Moreover, considering that ITI is costly and very demanding, novel approaches to ITI could be considered beneficial if they increased the proportion of successes, decreased the length of time to induce tolerance and/or decreased the cost of treatment. In this light, the use of immunomodulating and/or immunosuppressive drugs has been taken into account and increasingly implemented. Indeed these attempts are empirical, because as already discussed, the immunological mechanism that regulates immune tolerance toward FVIII is not fully understood yet.

The first experience of immunosuppressive therapy associated with ITI is represented by the Malmö protocol [48–50]. According to this protocol, if the inhibitor titer is above 10 BU/ml ITI is preceded by extracorporeal adsorption to protein A in order to reduce the titer below 3 BU/ml and cyclophosphamide is given from the first day, first intravenously 12–15 mg/kg/day for 2 days and then orally at 2–3 mg/kg/day for 8–10 days in order to reduce leukocyte count. Moreover, from Day 4 of treatment, IgG are given intravenously at 0.4 g/kg/day for 5 days [50]. This approach provided success rates similar to that obtained with ITI only (i.e., 67%), with

the only advantage of achieving rapidly a negative inhibitor titer and detectable levels of FVIII [50,51].

Since 2001 the use of rituximab as adjuvant therapy for ITI treatment has been reported. Rituximab is a chimeric human–mouse monoclonal antibody that reacts with CD20, a transmembrane protein expressed on B cells but not on plasma cells, that regulates the initial steps of cell-cycle activation and differentiation. Up to date, more than 50 patients with congenital hemophilia have been treated with rituximab in order to get rid of inhibitors, 42 of them being affected with severe hemophilia A [72–78]. All but three failed one or more previous courses of ITI [72–78] and the vast majority received rituximab as children or adolescents (i.e., 35 cases were younger than 18 years old; age range: 2–58) for one course of treatment consisting in a total of four doses in one month (i.e., four weekly doses). Only two patients repeated the whole course two- and three-times, respectively [74]. ITI with high-dose (i.e., 100–200 IU/kg/day) FVIII was associated in 30 patients [72–78]. A negative inhibitor titer was achieved in 2 out of 12 (17%) who received rituximab as unique therapy and in 21 out of 30 (70%) who received rituximab in association with ITI, nevertheless 10 patients received other immunosuppressive drugs together with rituximab. On the whole, 12 patients (29%) relapsed after 1 year of post-treatment follow-up and for many other the long-term outcome was not reported.

The use of regular intravenous immunoglobulins in association with high-dose FVIII daily infusion has been reported in two boys with severe hemophilia A and high-titre inhibitors who ultimately achieved tolerance [79,80]. However, one case had good predictors of response and could have been successful by ITI alone [79] and the second case interrupted prematurely the first attempt of ITI due to difficult venous access and underwent an ITI rescue course with the association of Ig, prednisone and azathioprine 5 years later [80].

The role of infusions frequency during ITI

With respect to ITI regimens lot of attention was paid to the dose used per injection rather than on the injection schedule (i.e., daily vs non daily). Indeed dose frequency may have a relevant role in tolerance induction as well as the dose used. Unfortunately in the I-ITI study doses and frequency of injections were strictly linked and could not be analysed separately [38].

How to define ITI outcome

Although established by consensus and widely accepted after their adoption by the I-ITI study [38], the laboratory parameters currently used to define ITI outcome are very strict rendering outcome figures not really reflecting the clinical gain obtained with

ITI. In fact, under a clinical point of view, partial responses are generally considered as successes considering that FVIII replacement is restored, although impaired PK usually leads to use FVIII concentrates at higher dose repeated at shorter intervals as compared with replacement therapy in non-inhibitor patients. Moreover, the need for repeating PK and inhibitor testing after defined time intervals and with the need of wash-out since last FVIII infusion makes all the assessment procedure quite demanding both for patients and clinicians. In addition, the current definition of failure implies that the minimum ITI duration is 9 months and the maximum is 33 months; however, the decision on ITI duration is often influenced by various and heterogeneous factors including clinical and prognostic features as well as economic evaluations and patients' quality of life. Finally, the occurrence of approximately 4–8% relapses after successful ITI reveals that these parameters are not 100% sufficient to define ITI response and other determinants of long-term maintenance of tolerance need to be investigated. With this respect, pragmatic definitions of success were recently proposed by UK Haemophilia Centre Doctors Organization [52]. In particular they consider tolerance achieved when:

- FVIII half-life is >7 h after a 72-h wash-out period or;
- FVIII trough level is $\geq 1\%$ 48 h after a dose ≤ 50 IU/kg (standard prophylaxis on alternate days).

The management of patients who failed ITI

The management of that 20–30% of patients who fail first line therapy is still committed to rescue treatment protocols. Apart from abandoning a second ITI attempt and continuing treatment with by-passing agents, second line options include one or a combination of the following:

- Increasing FVIII dose up to 200 IU/kg/day;
- Changing FVIII product type by switching from recombinant to plasma-derived concentrates (or vice versa) since up to now no superiority of one class over the other has been demonstrated and the role for VWF is only suggested;
- Adding immunosuppressive drugs.

However, none of the aforementioned strategy is so far supported by robust evidence coming from controlled clinical trials that are indeed needed in order to evaluate the best treatment strategies for these difficult-to-treat patients.

ITI in mild & moderate hemophilia

The UK Haemophilia Centre Directors Organisation reported an annual incidence of inhibitors of 0.84 per 1000 patients per year in mild and moderate hemophilia A as compared with 3.5 per 100 patients per year in severe ones [81]. More recent data from a large retrospective cohort study (i.e., the INSIGHT Study) showed a cumulative incidence of inhibitor of 5.3% in this group of patients [82]. Although rare, inhibitor development represents a clinical challenge also in mild and moderate patients since antibodies cross-react with endogenous FVIII reducing baseline levels often below 1 IU/dL and causing bleeding. At variance with inhibitors in severe patients, often inhibitors in mild hemophilia display type II kinetics similar to that of autoantibodies in acquired hemophilia. Owing to the ambivalent nature of alloantibodies against exogenous FVIII but cross-reacting against endogenous FVIII as autoantibodies, attempts at eradicating persistent inhibitors in mild hemophilia have been made both with various ITI regimens including the Malmö protocol [14,21,43,50,81–83] or by using immunosuppressive drugs with or without ITI [82,84,85], although up to 71% of these inhibitors tend to clear spontaneously [81,82,85]. A systematic review of the literature on the use of rituximab as treatment to eradicate inhibitors in congenital hemophilia reported on 16 patients with mild or moderate hemophilia A who received rituximab with success in 12 [84]. Another study reported on 32 cases of mild/moderate patients with inhibitors of whom 17 cleared the antibody after treatment [85]. However, survival analysis showed that treatment had no relevant impact on the probability of clearing inhibitors as compared with spontaneous clearance but on the time to disappearance that was shorter in patients who received treatment as compared with those who cleared the inhibitor spontaneously [85].

Currently, the identification of patients with mild/moderate hemophilia A at high risk of inhibitor development is the most important aspect in order to prevent inhibitor formation upfront; however, in case of inhibitor development both traditional ITI and immunosuppressive regimens may have a role for inhibitor eradication although predictors of success/failure of such therapies still need to be investigated. An analysis pertaining eradication strategies used in the frame of the INSIGHT Study is underway [86].

Cost–benefit & cost–effectiveness evaluation of ITI

Early, long-term, high-dose ITI regimens appear to be the most successful and convenient; however, costs of 1 million dollars per year for 1.5–2 years are not uncommon. Healthcare agencies are now carefully

scrutinizing health costs and they often expect data on clinical outcomes as well as on cost–benefit or cost–effectiveness of a given treatment before approving its use. Only scarce data exist with respect to the increased annual costs of factor replacement between inhibitor and non-inhibitor patients, and these data must take into account lifestyle, cost of care and orthopedic outcomes of patients with long-standing high-titer inhibitors as compared with non-inhibitor patients. In fact beside factor consumption, cost evaluation must include surgeries (e.g., central venous lines insertions), hospitalizations (e.g., central venous lines infectious/thrombotic complications), the need for emergency and routine visits and patients' productivity. Moreover, in order to evaluate which is the real burden of ITI in terms of cost–benefit or cost–effectiveness the definition of success is critical. By looking at the data from the IITR a significant difference in costs was found by comparing good and poor risk patients [87]; however, the criterion used to define the latter was only the adult age that *per se* is a weak predictor of response. Using available data from the published literature and estimates of the cost of relevant therapies and outcomes, a decision analysis model was constructed to describe the outcomes of two treatment strategies: successful ITI followed by FVIII replacement therapy versus the indefinite use of by-passing agents [88]. In this model the ITI regimen was 100 IU/kg/day and a success rate of 80% was assumed [88]. The model revealed that ITI is cost-saving and also clinically superior to long-term by-passing therapy [88]. Surprisingly, threshold analysis revealed that ITI was preferable over by-passing therapy even if success probability was set at 30%, that is, well below the predicted overall success [88]. Thus, despite the initial cost, over the course of a lifetime ITI resulted in net savings of \$1.7 million and 4.6 years increased life expectancy [88]. In this study only costs of factor concentrates and the cost of 1 year of prophylaxis following ITI completion were considered, assuming no additional clinical benefits derived from ITI, whereas the inclusion of the decreased disability associated with long-term prophylaxis as well as other costs related to the persistence of high-titer inhibitors would have likely further strengthen the results in favor of ITI. This example shows that expensive therapies for chronic diseases may be cost-effective if analyzed from a societal perspective over the patient lifetime. A similar modeling exercise was undertaken in the UK to evaluate the cost–effectiveness of treatment options for patients with hemophilia A and inhibitors [89]. In this model three ITI regimens (namely, the Bonn, the Malmö and the low-dose protocol) and a by-passing on demand regimen were considered [89]. The model showed that

the Malmö protocol is the preferred one since it generated more quality adjusted life-years and less costs [89]. Such result is related to the short duration of Malmö protocol, but it is noteworthy that this regimen is not widely used due to the need of immune adsorption and the concerns related to the use of cyclophosphamide in children. Interestingly, similar results were obtained by applying the same model in a different background population with different resource use [90]. Finally, another decision model was constructed from the perspective of the German statutory health insurance to evaluate treatment options for young children with severe hemophilia A and inhibitors. The model evaluated four treatment regimens: high-dose ITI, low-dose ITI, ITI based on risk assessment (i.e., identification of predictors of ITI outcome) and on demand treatment with by-passing agents [91]. Overall the third scenario resulted the cost-effective regimen with comparable outcomes to high-dose ITI but with a €0.7 million cost saving [91].

In the current healthcare scenario, decisions are often undertaken by policymakers using different perspectives and timeframes. Immediate economic pressures may discourage the use of expensive treatments for which cost savings do not pertain the short term; however, in the case of chronic diseases with time-related possible morbidity the choice of a long-term view and more global perspective is crucial.

In this light, the economic evaluation of the first and unique randomized clinical trial on ITI is eagerly awaited, owing to the fact that the success rate of the two treatment arms was similar but patients treated with the low-dose regimen achieved tolerance in a significant longer time period and presented a higher bleeding frequency especially in the first phase of ITI (i.e., from ITI start to negative inhibitor titer) [38]. Hopefully this evaluation will take into account the economic burden of the concomitant use of by-passing agents and FVIII during the first phase of ITI, usually neglected in the aforementioned economic models. So, it will reveal if time needed to achieve tolerance has a significant impact to factor consumption and how great is the burden of treatment-related morbidity. Moreover, such evaluation could suggest the opportunity of using different treatment schedules over time (i.e., daily versus non-daily regimens) according to the main milestones of ITI (i.e., negative inhibitor titer and PK normalization) considering that usually they occur separately and often with a considerable time interval in between [38].

ITI & hemophilia B: data from clinical observation

Inhibitor development is much rarer in hemophilia B than in hemophilia A patients (1–3% vs 20–30%) [92].

This difference is in part related to the fact that patients with large deletions or nonsense mutations are at the highest risk; however, these mutations are quite rare in hemophilia B patients being missense mutations the most common gene defects [93–98].

Although very rare, inhibitor development in hemophilia B can be a big clinical challenge since some patients can experience severe allergic or anaphylactic reactions to FIX infusions prior to, simultaneously or soon after the appearance of inhibitors and/or may develop nephrotic syndrome after repeated FIX infusion as for ITI [99–111]. Such complications render ITI treatment challenging or unfeasible, leading to very low success rates, mainly related to the fact that the vast majority of treating physicians simply do not use ITI. Moreover, the presence of FIX in the aPCC prevents its use in these patients, so limiting treatment possibilities to rFVIIa in case of bleed. Due to the rarity of hemophilia B complicated by inhibitors the experience with ITI in this setting is limited and consequently published data are scarce and mainly described as small series or case reports.

The Malmö protocol was first conceived and used for inhibitor hemophilia B patients with good results; however, none of those patients had had allergic reactions to FIX [48,49].

Due to the possibility of anaphylaxis or in patients who already experienced allergic reactions, usually the first step of treatment is a desensitization protocol in order to abolish such reactions to FIX. This has been attempted by gradually increasing the dose of FIX concentrate given intravenously or subcutaneously [99,108,112], by using slow intravenous infusion of FIX concentrate [100,108,112], or by eliminating the antibody from bloodstream with plasmapheresis [102,103]. Similarly, different approaches have been undertaken to perform ITI. Usually FIX is given at high daily doses [99,100,104,107,109,110,112–114] but attempts with lower dose have been reported as well [100,105,109,110], the Malmö protocol was adopted in some cases often omitting immunoadsorption [99,100,112], continuous FIX infusion was used in one case [115] and, more recently, various immunosuppressive drugs as mycophenolate or rituximab have been added to treatment protocols with or without parenteral Ig and steroids [78,106–108,111,113,116–120]. Nephrotic syndrome usually occurs 8–10 months after ITI start and prevents ITI continuation irrespective of the result obtained [99–101,112].

Due to the limited number of cases reported, it is not possible to evaluate which treatment regimen is associated with the better outcome. Moreover adverse reactions can heavily affect the success rates that are rather low, not exceeding 30% as reported in the NAITR [11].

ITI & hemophilia B: what still needs to be investigated

Feasibility of ITI in hemophilia B patients with inhibitors still need to be optimized; however, the major limit is represented by the fact that the immunologic mechanism underlying the immune response against FIX is still unknown. Considering the clinical symptoms, it is assumable that an IgE-mediated reaction is triggered and complement activation products are involved; however, this has never been demonstrated so far.

Similarly, the pathogenesis of nephrotic syndrome remains unclear. Renal biopsy revealed histology consistent with membranous glomerulonephritis in two cases; however, no glomerular FIX deposition was detected [101,111].

Due to the peculiar behaviour of inhibitors in hemophilia B patients, the use of immunosuppressive therapies that may produce a rapid clearance of the antibody from the circulation is quite attractive even if scarcely accomplished so far. The use of rituximab for inhibitor eradication in patients with hemophilia B with inhibitors who failed ITI have been reported for a total of 10 patients [78,106–108,111,116–120]. Undetectable inhibitor titer was obtained in seven patients after a median of 2.5 months and relapse was reported only in one case [118]. The two nonresponders did not receive FIX concomitantly and in three successful

cases other immunosuppressive drugs were associated [106,111,119]. Although a number of good responses have been reported, they are all single case reports, whose analysis does not allow to draw any conclusion and further studies are needed to ascertain long-term safety and efficacy of these therapeutic approaches.

Conclusion

Inhibitor development still represents the major clinical challenge in the management of patients with hemophilia. Inhibitor eradication is the only way to significantly impact on the morbidity related to such complication. Immune tolerance induction treatment is high but not fully successful at this aim since several predictors of success and failure interact in the same patient. In this light future studies need to be carried out in order to optimize and tailor this treatment strategy in different patient groups.

Future perspective

ITI was introduced nearly 30 years ago. Despite its successful clinical application, still there is no clear understanding of the mechanisms responsible for the downregulation or abolishment of the established anti-FVIII antibody response and the induction of long-lasting immune tolerance. Currently none of the single immunomodulation protocols (i.e., immunosuppressive drugs, anti-idiotypic antibodies,

Executive summary

Background

- Inhibitor development is the main complication of hemophilia replacement therapy.
- Inhibitors occur in 25–30% of children with severe hemophilia A and 2–5% of those with severe hemophilia B.
- In the presence of high-titer inhibitors standard replacement therapy is ineffective, hence the control of bleeding episodes is difficult and prophylaxis, aimed at preventing joint damage, is unfeasible.
- In the presence of inhibitors, patients are prone to develop chronic progressive joint damage that often cause physical disability.

Immune tolerance induction: what is known

- Immune tolerance induction (ITI) treatment is the only therapeutic strategy able to eradicate persistent high-titer inhibitors with a success rate of 60–80%.
- Many patient- and treatment-related predictors of ITI response have been identified since the first report of ITI in late 1970.
- High success rates have been obtained with heterogeneous treatment schedules.
- ITI is demanding both for patients/parents and for clinicians because it is often accomplished with daily infusions of high-dose FVIII/FIX.
- ITI is a costly treatment and this aspect limits its affordability worldwide.
- ITI optimization is essential in order to tailor treatment on the basis of predictors of response and to better allocate available resources.

ITI: what needs to be investigated

- The main aspect of ITI that still needs to be investigated pertains the identification of the immunological mechanisms that regulate immune tolerance.
- Other major open issues in this field regard the definition of ITI response with respect to short- and long-term outcomes, ITI in patients with hemophilia B, ITI in patients with mild/moderate hemophilia, the use of immunosuppressive drugs as adjuvant therapies and the role of von Willebrand factor.
- A detailed pharmaco-economic assessment of ITI should be included in future studies.

B-cell depleting antibodies) that have been investigated is able to eradicate inhibitors and induce long-term tolerance to FVIII without ITI. A combination therapy that targets different pathways is most likely to be successful. The ideal strategy should include the depletion of memory B and/or T cells and/or long-lived plasma cells to eliminate pre-existing immune response combined with the expansion of antigen-specific Treg cells to maintain long-term T-cell hyporesponsiveness to FVIII. Properly designed clinical trials that include an extensive analysis of the immune system during the whole course of ITI are awfully needed; however, due to the rarity of these patients only a joint international/multinational collaboration could allow such a study.

While exploring these aspects of basic research, the clinical optimization of standard ITI protocols is eagerly needed and at this aim it is needed to:

- Identify patient characteristics associated with ITI response that may help to draw risk profiles for success and/or failure of ITI;
- Identify patient and/or treatment characteristics related to high chances of ITI success and to the maintenance of long-term tolerance;

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- Better explore the long-term safety and efficacy of immunosuppressive drugs in the frame of multicenter controlled clinical trials;
- Evaluate the cost–effectiveness of ITI considering the long-term outcome of joint status of patients who achieved success in order to appreciate the concrete result of the often relevant economic investment done to afford huge amount of concentrate for a single course of successful ITI as currently defined;
- Revise the definitions of success on the basis of the results of a long post-treatment follow-up.

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