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Functional polymorphisms in the *LDLR* and pharmacokinetics of Factor VIII concentrates

Running head: Factor VIII Pharmacokinetics and *LDLR* SNPs

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Essentials

- The low-density lipoprotein receptor (LDLR) might participate in factor VIII (FVIII) clearance.
- *LDLR* polymorphisms and FVIII pharmacokinetics (PK) have been assessed in patients with ~~in~~ hemophilia A (HA).
- *LDLR* c.1173C/T and *ABO* alleles were similarly and independently associated with FVIII PK.
- Data support both *LDLR* c.81C/T and c.1173C/T as genetic determinants of FVIII PK in HA patients.

Summary. *Background*: Optimization of factor VIII (FVIII) infusion in hemophilia A (HA) would benefit from identification of FVIII pharmacokinetics (PK) determinants. The low-density lipoprotein receptor (LDLR) contains a FVIII-binding site and might influence FVIII clearance. Consistently, *LDLR* polymorphisms have been associated with FVIII levels. *Objective*: To investigate the relationships between individual FVIII PK and functional *LDLR* polymorphisms. *Patients/Methods*: Thirty-three HA patients (FVIII:C ≤ 2IU/dL) without inhibitors underwent 85 FVIII single dose (21.4-51.8 IU/kg) PKs with different FVIII concentrates. Twenty patients underwent repeated PKs (2-6). FVIII:C measured up to 72 hours was analyzed by Two-Compartment Model. ~~Final and secondary~~ Parameters were evaluated in relation to *F8* mutations, *ABO* blood-group and *LDLR* genotypes. *Results*: *F8* mutation types were not associated with PK parameters. *ABO* and *LDLR* c.1773C/T polymorphism were associated with Alpha, Alpha HL, CLD2, K1-2 and K2-1 parameters, suggesting an influence on the FVIII initial distribution phase. Regression analysis showed an independent association of both *ABO* and *LDLR* c.1773C/T with PK parameters (Alpha, ß-coefficient -0.311 vs 0.348; CLD2, ß-coefficient -0.335 vs 0.318), giving rise to an additive effect ~~with a gradient of values~~ in subjects stratified by combined phenotypes. Differently, the *LDLR* c.81C/T was associated with FVIII clearance and volume of distribution at

steady state, which could be related to distinct effects of polymorphisms, potentially linked to LDLR intracellular distribution and FVIII binding behavior. *Conclusions*: With the limitation of different FVIII concentrates and low number of patients, our data show plausible associations of *LDLR* polymorphisms with FVIII PK parameters, thus supporting their investigation as candidate functional determinants of FVIII PK.

Keywords: *ABO* blood-group; factor VIII; hemophilia A; *LDL-receptor* polymorphism; pharmacokinetics.

Introduction

The increasing importance of prophylactic treatment in patients with hemophilia A (HA) to prevent joint damage [1,2] makes individualization of prophylactic dose a key issue to optimize factor VIII (FVIII) prophylaxis strategy. On the other hand, several studies support a large inter-patient variability [3-5] of FVIII pharmacokinetics (PKs), which poses a challenge for optimal treatment with FVIII products, and supports the investigation of the main determinants of PK. Among these, polymorphisms in genes participating in the several biological steps leading to FVIII clearance represent natural candidates.

Genome wide analyses of endogenous FVIII levels have indicated, together with *ABO* and von Willebrand factor (*VWF*) loci, several other potential modifiers, *SCARA5, STAB2, STXBP5* [6] and *KNG1, MAT1A, TMLHE* [7], which could be involved in the FVIII biosynthetic process. The *ABO* blood-group, a master locus in post-translational glycosylation, explains about 10-20% of the variation in FVIII:C levels [8,9].

ABO blood-group, VWF and patient's age [10-12], among established FVIII modifiers, are candidate to influence FVIII PK parameters, and particularly half-life, although they might explain only a small portion of the PK variability. Of note, FVIII-binding IgG modulates FVIII half-life in patients with severe and moderate HA without inhibitors [13], which adds further complexity to this field.

Among FVIII receptors, the low-density lipoprotein receptor-related protein 1 (LRP1), a member of the LDL receptor family of endocytic receptors, has received particular attention in light of previous data on animal models [14,15], healthy subjects and patients with venous thrombosis [9,16]. However, no correlation was found between *LRP1* genotypes/LRP1 levels and FVIII PK parameters, particularly clearance and half-life [17].

Altogether these findings suggest that several genetic and acquired components, with partially overlapping effects on both endogenous and infused FVIII, might act in combination to regulate bio-distribution and clearance of FVIII molecules.

Among these, the low-density lipoprotein receptor (LDLR), the key member of the family of endocytic receptors, represents a noticeable candidate because of biochemical, animal model-related and epidemiological evidences. The region of complement-type repeats 2–5 in LDLR was defined as the binding site for FVIII [18] and LDLR has been involved in FVIII clearance in animal models [19], in which LDLR deficiency prolonged the half-life of FVIII. In humans, we have reported that single-nucleotide polymorphisms (SNPs) in the *LDLR* gene independently associated with high plasma levels of FVIII in patients with coronary artery disease (CAD) [20,21]. Interestingly, Gao et al [22] reported that two polymorphisms, located in different exons of *LDLR,* influence the receptor expression by distinct mechanisms.

Here, we investigated the relationships between the outcomes of a large number of individual PKs in HA and key polymorphisms in the *LDLR* gene, to test the hypothesis that gene variation, regulating the expression of the *LDLR,* could also influence permanence in plasma of infused FVIII.

Methods

Study design and Patients

The pharmacokinetic (PK) study of plasma-derived (pd-FVIII) and recombinant FVIII (r-FVIII) concentrates was conducted in the 2010-2013 period at the Comprehensive Hemophilia Care Center, Careggi University Hospital, Florence, Italy. The *F8* and *LDLR* were genotyped at the Genetic Diagnostics Unit, Careggi University Hospital (Florence, Italy) and at the Laboratory of

Molecular Biology of Haemostasis, University of Ferrara (Ferrara, Italy) respectively. All enrolled patients expressed their oral informed consent for PK execution and written for genotyping.

Thirty-three HA patients (FVIII:C ≤ 2IU/dL) without inhibitors underwent a single dose PK with pd- and/or r-FVIII. Twenty-three patients underwent PK with high purity (HP) pd-FVIII, 12 with B domain deleted (BDD) r-FVIII, 11 with full length (FL) r-FVIII and 5 with intermediate purity (IP) pd-FVIII. Twenty patients (61%) underwent repeated PKs (2-6).

After a 3-4 days wash out, the patients were infused with a single dose of FVIII (21.4-51.8 IU/kg) and the blood samples were collected before infusion and after 0.25, 0.5, 1, 3, 6, 9, 24, 28, 48 and 72 hours.

Plasma assays

Platelet poor plasma was stored at -40°C in 0.5 mL aliquots. FVIII coagulant activity (FVIII:C) assays were done at the same time, by One-Stage Method, on three duplicate dilutions of baseline and post-infusion samples according to the previously described method [23]. Reagents and instrumentation were from Dade Behring (Deerfield, IL, USA) as follows: rabbit brain partial thromboplastin and ellagic acid (Actin FS); immunodepleted Factor VIII deficient plasma; commercial FVIII plasma standard calibrated against WHO-standard; Imidazole buffer, pH 7.35; Calcium chloride, 0.025 M; Coagulometer Behring Coagulation Timer (BTC). VWF antigen (VWF:Ag) determination was performed as previously described [24].

PK methods

When the FVIII concentration at baseline was > 2 IU/dL, due to an ~~incorrect~~ insufficient wash out, the FVIII/time concentrations of each PK were corrected according to the Bjiorkman formula [12]. Afterward, each PK decay was analyzed by One-Compartment and Two-Compartment

Models (OCM and TCM respectively) by WinNonlin. The data of 85 PKs fitted the TCM (Table S1). As index of the best fitting iterative procedure, WinNonlin provided us with the Akaike Information Criterion (AIC), the Schwartz Bayesian Criterion (SBC) and the Sum of Squared Residuals (SSR). According to these three concordant indexes, 80 PKs among the original 85 PKs resulted fitting better the TCM. Accordingly, we considered final and secondary PK parameters from 80 PKs as follows:

Final parameters:

- K 1-0, elimination rate from the central compartment
- K 1-2, transfer rate from central (1) to peripheral (2) compartment
- K 2-1, transfer rate from peripheral (2) to central (1) compartment
- V1, Volume of central compartment

Secondary parameters:

- At zero time extrapolated FVIII concentration (C max)
- Area under the curve (AUC)
- The Moment of AUC (AUCM)
- Alpha rate constant associated with the distribution phase (Alpha)
- Alpha distribution half-life (Alpha HL)
- Beta rate constant associated with the elimination phase (Beta)
- Beta elimination half-life (Beta HL)
- Clearance (Cl)
- Inter-compartment clearance (CLD2)
- Mean Residence Time (MRT)
- Volume of peripheral compartment (V2)
- Volume of distribution at steady state (Vss)

Polymorphisms and genotyping

Genotyping for intron 22 inversion (IVS 22) of *F8* and for *ABO* blood-group was performed as previously described [9,25]. *F8* mutations were detected by direct sequencing [26].

Three polymorphisms of *LDLR* gene (c.81C/T, c.1171G/A, c.1773C/T, ref. NM_000527.4) in exon 2, 8 and 12, and codifying for p.Cys27, p.Ala391Thr, p.Asn591 (ref. NP_000518.1) respectively, were selected (Table S2). The SNPs of exons 2 and 12 were detected by *BstUI* and *HincII* restriction analysis respectively, as previously described [20]. The exon 8 (c.1171G/A, p.Ala391Thr) polymorphism was detected by direct sequencing with the following primers (forward 5'-ACCTGGCTGTTTCCTTGATTAC-3', reverse 5'-AGAGCCCTCAGGAGCAAACAG-3').

Statistical analysis

The PK analysis was performed ~~by means of the gold standard software,~~ using WinNonlin 7.0 (Phoenix 64, Pharsight), available by courtesy of the Italian Association of Hemophilia Centers (AICE). For each patient (n = 20) with repeated PKs (total number = 67) mean values were calculated and listed together with those of the 13 patients with a single PK. In total we collected 33 PK outcomes and the final and secondary PK parameters were used for association studies with genetic polymorphisms.

All statistical analyses were performed using IBM® SPSS® Statistics version 23.0 software (IBM Corp. Armonk, NY, USA) and figures were produced by Graphpad prism version 5.00 (GraphPad Software, Inc. La Jolla, CA, USA).

Distributions of continuous variables (PK parameters) in groups were expressed as mean and 95% confidence interval (95% CI). Variables showing a skewed distribution (i.e. K 1-0, K 1-2, K 2-1, V1, Alpha, Alpha HL, Beta, Beta HL, Cl, CLD2, C max, V2, Vss, AUMC) were logarithmically

transformed and their analyses performed on log-transformed values. Genotype-related differences in PK parameters were analyzed by t-test or analysis of variance (ANOVA), ~~when indicated~~ as appropriate. The results of *LDLR* c.1773C/T polymorphism were adjusted for *ABO* blood-group by linear regression analysis. Finally, taking into account the additive effect on Alpha and CLD2 PK parameters of *ABO* blood-group and *LDLR* c.1773C/T, the study population was stratified on the basis of their combined genotypes, and Alpha or CLD2 data were analyzed across the obtained 6 subgroups by ANOVA with polynomial contrasts for linear trend.

Results

The characteristics of the 33 HA patients (FVIII:C ≤ 2 IU/dL) undergoing the FVIII PK are reported in the Table S3.

~~Table S1 reports the indexes of best-fitting analysis of OCM compartmental analysis compared to TCM. According to coefficient regression of observed and predicted FVIII concentration/times, we used only the TCM for evaluating the relationships between the PK outcomes of infused FVIII concentrates and patients' genotypes.~~

Taking into account that different FVIII concentrates were used, we preliminary compared the outcomes of PK grouped by type of concentrates. The PK from FL pd- and r-FVIII concentrates (n = 65) were compared with BDD FVIII concentrates (n = 15). No significant differences were found. Intermediate purity (IP) pd-FVIII PK (n = 8, 5 patients) was compared with high purity (HP) pd-FVIII PK (n = 33, 23 patients). No significant difference was observed, even though a trend for lower Alpha values in IP pd-FVIII was present (P = 0.10). Further comparison of concentrates is reported in the "Association of PK outcomes with LDLR gene polymorphisms" section.

Association of PK outcomes with type of F8 mutations and ABO blood-group

Patients were grouped according to type and frequency of *F8* mutations. Twelve (36%) patients were affected by the IVS 22 inversion, 11 (33%) by missense mutations and 5 (15%) by deletions/insertions. The most represented mutation groups were compared for values of PK parameters. For the association analysis, patients with IVS 22 inversion (n = 12) were firstly compared with the other patients (n = 21). Secondly, patients with missense mutations (n = 11) were compared with those with null mutations (n = 17), 12 with IVS 22 inversion and 5 with insertions/deletions. We did not observe differences in PK parameters in relation to FVIII genotype

groups (Table S4). However, a border-line significant trend was observed for the presence/absence of the IVS 22 inversion in relation to the final parameter K 2-1 (Table S4).

Genotyping for *ABO* blood-group revealed that 23 patients (70%) were non-O. The results of the PK analysis in relation to these genotypes are reported in Table 1. The O blood-group was associated with higher K 1-2 (P = 0.043), K 2-1 (P = 0.016), higher Alpha (P = 0.015) and CLD2 (P = 0.012) than non-O group. We also observed a trend for shorter Alpha HL (P = 0.051), and lower C max (P = 0.055), AUC (P = 0.058) and AUCM (P = 0.057). The mean FVIII:C decay curves in O and non-O blood group patients are shown as supplementary material (Fig. S1A).

Association of PK outcomes with LDLR gene polymorphisms

Three *LDLR* polymorphisms, extensively investigated for their association/ability to influence receptor expression and FVIII levels in CAD patients, were genotyped and analyzed in relation to the PK parameters.

The analysis of PK values according with *LDLR* c.1171G/A genotypes (GG, n = 29; GA, n = 4) did not show significant differences.

On the other hand, PK parameters of patients grouped by the *LDLR* c.1773C/T genotypes (CC, n = 8; CT, n = 20 and TT, n = 5) displayed significant differences by comparing the three genotypes with ANOVA, namely for the K 1-2 (P = 0.043), K 2-1 (P = 0.034), Alpha (P = 0.011), Alpha HL (P = 0.019) and CLD2 (P = 0.016) (Table 2 and Fig. S2). The comparison of TT homozygotes (n = 5) with homozygotes and carriers of the C allele (C-carriership, n = 28) produced significant differences for Alpha (P = 0.042), Alpha HL (P = 0.017) and CLD2 (P = 0.030). The comparison of specific genotype groups confirmed the Alpha, Alpha HL and CLD2 differences between the c.1773 CC and TT (P = 0.022, P = 0.021 and P = 0.033 respectively) and between the CT and TT genotypes (P = 0.033, P = 0.037 and P = 0.065 respectively). To exemplify mean genotype-

associated differences the mean FVIII:C decay curves relative to the CT and TT genotypes are also reported as supplementary material (Fig. S1B).

We also evaluated the effect of removing groups of patients (treated with specific concentrates) from the association analysis of PK parameters. For example, 11 (33%) patients used the FL r-FVIII concentrates. However, the distribution of values and the association in relation to the LDLR genotypes were substantially maintained in the remaining 22 patients (CC, n = 5; CT, n = 13 and TT, n = 4). For the Alpha, Alpha HL and CLD2 parameters, the Alpha was 1.02 (0.00-2.07); 1.66 (0.67-2.65) and 4.50 (0.00-10.73) respectively (ANOVA, P = 0.072). These values were comparable with those reported in Table 2. Further, a comparable distribution of the *LDLR* c.1773 CC, CT, TT genotypes (FL r-FVIII n = 3, 7, 1 *vs* patients using other products n = 5, 13, 4) was indicated by Chi-square analysis (P = 0.78).

Taking into account that *ABO* blood-group showed association with the same PK parameters, an adjustment analysis was performed by including *ABO* and *LDLR* c.1773C/T polymorphism in linear regression models with PK parameters as dependent variables (Table 3). Both *ABO* and *LDLR* c.1773C/T polymorphism showed independent and comparable influence on phenotypes. More precisely, as regards Alpha, P values remained significant for the *LDLR* c.1773C/T (P = 0.040, β coefficient 0.348) as compared with a trend for *ABO* (P = 0.065, β coefficient -0.311). Conversely, the influence of *ABO* genotypes was significant on CLD2 (P = 0.049, β coefficient -0.335), as compared with a trend for *LDLR* (P = 0.060, β coefficient 0.318). Stratifying the study population on the basis of *ABO* and *LDLR* c.1773C/T (Fig. 1), a gradient shape for Alpha and CLD2 parameters was observed with values increasing progressively from non-O – CC to O – TT subgroups (P = 0.003 by ANOVA with polynomial contrasts for linear trend).

Differently from the *ABO* and *LDLR* c.1773C/T, the *LDLR* c.81C/T genotypes were associated with significant differences in Cl (P = 0.047) and in Vss (P = 0.033) parameters (Table S5 and Fig. S1C).

Carriership analysis (T-carriers, n = 9) confirmed these differences (Cl, P = 0.025 and Vss, P = 0.027) (Fig. 2).

Inter- and intra-patient variability of PK parameters

For most patients, more than one PK was available, which permitted us to use in the genotype association analysis the mean values of PK parameters. In patients with at least three PKs (n = 12), we evaluated (Table S6) the mean intra-patient coefficient of variation (CV) for PK parameters found to be significantly correlated with *ABO* and *LDLR* c.1773C/T polymorphism (Alpha, Alpha HL and CLD2) or with *LDLR* c.81C/T polymorphism (Cl and Vss). Inter-patient variability was also estimated for the whole cohort of patients, and for groups undergoing a single PK or more than one PK (Table S6).

VWF levels, LDLR c.1773C/T polymorphism and PK parameters

Mean VWF:Ag levels, available in 31 out of 33 patients, were 130 ± 33 IU/dL in non-O genotypes and 122 ± 26 IU/dL in the O blood-group. VWF:Ag levels were not associated (P = 0.493) with the *LDLR* c.1773C/T polymorphism (CC n = 7, 128 ± 40 IU/dL; CT n = 19, 124 ± 30 IU/dL; TT n = 5, 141 ± 20 IU/dL). VWF:Ag levels did not correlate with PK parameters associated with *ABO* and *LDLR* c.1773C/T polymorphism (Alpha, P = 0.482; Alpha HL, P = 0.755 and CLD2, P = 0.608).

Discussion

We hypothesized that genotypes influencing the expression of *LDLR*, a receptor with the ability to bind FVIII molecules, and of endogenous FVIII levels in CAD patients could also be candidate to modulate distribution and clearance of FVIII infused in HA patients. With this premise, we investigated the relationships of functional and frequent *LDLR* genotypes with FVIII PK parameters in patients, selected for having severe or moderately severe FVIII deficiency, who represent the population of patients frequently treated with FVIII concentrates.

PK FVIII doses and frequency of sampling were according to the recommendations of ISTH, FVIII/IX SSC [27,28], and the PK analysis was performed ~~by means of the gold standard software~~ using WinNonlin.

When grouped in relation to frequent and informative genetic features (*F8* IVS 22 inversion yes *vs* not, null *vs* missense mutations), *F8* genotypes failed to display any association. This lack of association suggests that the modest amounts of endogenous FVIII molecules produced by mutated *F8* genes, although of great importance for patients' bleeding phenotype, poorly influence the PK of infused products.

We found a number of plausible associations between PK parameters and genetic variations. The genotypes of the *ABO* blood-group, a known determinant of VWF [29] and FVIII half-life [10,17,30-33], were found to be significantly associated with a number of final and secondary PK parameters (K 1-2, K 2-1, Alpha and CLD2), and additional trends of association were detectable (Alpha HL, C max, AUC and AUCM). The association with Alpha rate constant suggests that the initial distribution phase is modulated, possibly *via* VWF, the main FVIII carrier. The K 1-2 and K 2-1 values, higher in the O patients, as well as the inter-compartment clearance, support that, after the first rapid flow from plasma to peripheral compartment, a compensatory flow back would start. The trend for lower C max, AUC and moment of AUC in O patients are in agreement with the initial less favorable response to infusion of FVIII concentrates in these patients. Overall, non-O

genotypes predicted better the decay curves, in accordance with previous data in HA patients [10,17,30-33].

Concerning the *LDLR* genetic variation, several and novel associations were detected. Noticeably, parallel influence of the *ABO* and the *LDLR* c.1773C/T polymorphism was observed, and in particular on K 1-2 and K 2-1, indicating that both the transfer rates are modulated by genetic variation, and on Alpha, Alpha HL and CLD2 parameters. The Alpha HL appeared to be negatively modulated by the T allele of the c.1773C/T polymorphism, and was shorter in the-TT homozygotes than in C-carriers. Conversely, the lowest Alpha rate constant was observed in the CC homozygotes. The transfer rate from central to peripheral compartment (K 1-2) of CC was lower than in the T-carriers, and compatible with smaller Alpha rate distribution constant and longer Alpha HL. The transfer rate from peripheral to central compartment (K 2-1) was higher in TT than in C-carriers in accordance with inter-compartment clearance (CLD2). This would imply that, after the initial flow from plasma to extravascular compartment, the system tends to a new equilibrium.

The regression analysis indicated that both the *LDLR* c.1773C/T and *ABO* polymorphism appeared to influence the initial distribution phase of FVIII, with comparable strength. Importantly, we observed additive effects, giving rise to a gradient of PK values in patients grouped by combined phenotypes. Taking into account the well-recognized role of the *ABO* blood-group on hemostasis and in FVIII PK [33], finding similar effects on PK exerted by a functional *LDLR* polymorphism is a noticeable observation.

Association with PK parameters differed in the *LDLR* c.81C/T and *LDLR* c.1773C/T genotypes. Indeed, the clearance from central to peripheral compartment was higher in the c.81CC homozygotes as compared with T-carriers. Accordingly, the volume of distribution at steady state was higher in CC-homozygous patients.

Differences in PK effects, exerted by polymorphisms located in the same gene, deserve discussion about their molecular effects on *LDLR* expression in light of previous molecular findings [22].

Interestingly, both the *LDLR* c.81C/T (rs2228671) and *LDLR* c.1773C/T (rs688) transitions, which are not in linkage disequilibrium [20], affect synonymous codons and result in changes from frequent to rare codons, that in turn could influence co-translational folding and hence LDLR function. The *LDLR* c.1773C/T has effect on LDLR activity beyond its role in alternative splicing [34], due to impairment of LDLR endosomal recycling and/or proprotein convertase subtilisin/kexin type 9 (PCSK9) binding [22]. Thus the *LDLR* c.1773C/T regulates post-transcriptionally *LDLR* both by exon 12 alternative splicing nonsense-mediated mRNA decay, and by altering intracellular receptor distribution. Differently, the *LDLR* c.81C/T would not alter PCSK9 binding [22]. These distinct effects might support differences in FVIII binding behavior and thus in FVIII PK modulation.

We are conscious that our study has some limitations, and among these the different FVIII concentrates used in the patients. It is worth noting that, concerning the FL and BDD FVIII products, the LDLR binds a FVIII epitope involving multiple Lysine residues located in the A3, C1 and C2 domains [18], and thus outside the B domain. Finding that the genetic associations were substantially maintained by analysis of FVIII concentrates, would suggest that the genetic components on PKs, both *ABO*- and *LDLR*-related, exert quite robust effects. At the same time, this permitted us the cumulative analysis of PKs of both FL and BDD products and thus to recruit a reasonable number of patients for PK analysis, according to the SSC recommendations [28]. An additional limitation is that the genotyping was conducted in a low number of patients, which does not favor the analysis of determinants in combinations. However, meaningful and additive effects of *ABO* and *LDLR* genotypes were observed. Nevertheless our data need to be confirmed in larger studies using single FVIII concentrates before suggesting their use to tailor FVIII prophylactic regimen. The comparison of genetic and acquired conditions influencing FVIII PK, which will permit to address the influence of several non-genetic factors on PK parameters, would also require an ample international cooperative study. ~~iv) VWF levels were available only in 25 out of 33 (75%)~~

~~patients. In these subjects, VWF antigen levels were slightly higher (123 ± 31 IU/dL) in non-O genotypes than in the O blood-group (116 ± 21 IU/dL).~~

For most FVIII PK parameters, the intra-patient variability was lower that inter-patient variability, which supports the investigation of genetic components. We also observed that the inter-patient variability was lower in patients with repeated PKs, which suggests that the use of mean PK values probably decreased inter-patient variation and might favor the investigation of genetic components. A large portion of FVIII PK variance is at present unexplained and, among the potential genetic determinants which are thought to interact with acquired conditions, we addressed our attention toward a plausible candidate, the *LDLR* locus. The specific *LDLR* genetic variation (c.1773C/T) that we report associated to FVIII PK has been previously reported by us [20] to influence the high FVIII levels present in patients with CAD. Although HA patients genetically experience low FVIII levels, the treatment with high concentrations of FVIII transiently exposes HA patients to high peaks of circulating FVIII, comparable to those of patients with cardiovascular disease. This could favor the interaction between FVIII and the LDLR, mediated by four adjacent complement type repeats (CR. 2-5) forming a binding site for FVIII with noticeable affinity (40-90 nM) [18]. However, this affinity could be potentially insufficient to bind efficiently the low concentration (0.3 nM) of FVIII in plasma. Thus, significant binding between FVIII and LDLR would require, in addition to increased FVIII levels present in cardiovascular disease or after infusion in HA patients, heparan-sulfate like proteoglycans, whose role for FVIII binding is still undefined. Further, apoB-100 and apoE-containing lipoproteins, in light of their tight binding of the LDLR [35,36], could modulate the inferred LDLR-FVIII interaction, which requires further studies.

Our results, reporting plausible associations of FVIII PK parameters with well characterized and potentially functional *LDLR* polymorphisms, foster further investigation in this field and support the *LDLR* variation as a candidate determinant of FVIII PK.

Addendum

F. Bernardi and M. Morfini designed research. S. Linari, G. Castaman, and M. Morfini recruited patients. M. Morfini performed the PK analysis. S. Frusconi performed *F8* sequencing and genotyping for *F8* IVS22 inversion. B. Lunghi genotyped *ABO* blood-group and *LDLR* polymorphisms. B. Lunghi, F. Bernardi, A. Branchini, G. Marchetti, N. Martinelli and M. Morfini analyzed and interpreted data. N. Martinelli, B. Lunghi and M. Morfini performed statistical analysis. B. Lunghi, F. Bernardi, N. Martinelli, G. Marchetti, A. Branchini and M. Morfini contributed to data discussion. B. Lunghi, F. Bernardi, N. Martinelli and M. Morfini wrote the manuscript.

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Disclosure of Conflict of Interests

F. Bernardi reports grants from Bayer, during the conduct of the study; grants from Pfizer, outside the submitted work. A. Branchini reports grants and personal fees from Pfizer, grants from Bayer, grants and non-financial support from Grigols, outside the submitted work. G. Castaman reports personal fees from Roche, personal fees from Bayer, personal fees from Shire, grants and personal fees from CSL Behring, grants and personal fees from Sobi, personal fees from Uniqure, grants from Pfizer, personal fees from Kedrion, personal fees from Novo Nordisk, personal fees from Werfen, outside the submitted work. M. Morfini reports personal fees from Kedrion, grants from

Pfizer, personal fees from Novo Nordisk, personal fees from SOBI, personal fees from Bayer, personal fees from Bioverativ, personal fees from Octapharma, personal fees from CSL Behring, outside the submitted work. The other authors state that they have no conflict of interest.

Legend to figures

Fig. 1. PK parameters (Alpha, 1A; CLD2, 1B) in relation to the *ABO* blood-group and *LDLR* c.1773C/T combined genotypes. Data (logarithmically transformed, geometric means) were analyzed across the 6 subgroups by ANOVA with polynomial contrasts for linear trend.

Fig. 2. PK parameters in relation to the *LDLR* c.81C/T carriership. A) Cl; B) Vss. The mean values and 95% CI are indicated. P, ANOVA of skewed variables logarithmically transformed. ○ = TT homozygous patient.

Supporting Information

Table S1. Best-fitting analysis of compartmental analysis.

Table S2. List of *LDLR* SNPs and detection methods.

Table S3. Characteristics of hemophilia A patients.

Table S4. PK parameters in relation to *F8* mutations.

Table S5. Analysis of association between PK parameters and the *LDLR* c.81C/T polymorphism.

Table S6. Inter- and intra-patient variability of PK parameters.

Fig. S1. Mean FVIII:C decay curves in patients grouped for genotypes. A) O and non-O blood group; B) *LDLR* c.1773 TT *vs* CT; C) *LDLR* c.81 CC *vs* CT.

Fig. S2. Alpha HL in relation to the *LDLR* c.1773C/T genotypes. The mean values and 95% CI are indicated. P, ANOVA of skewed variable logarithmically transformed.

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Table 1 PK parameters in relation to *ABO* blood-group

	***ABO* genotype**		
	O (n = 10)	non-O (n = 23)	P
Final PK parameters			
K 1-0 (1/hrs)	0.12 (0.03-0.20)	0.08 (0.06-0.10)	0.280
K 1-2 (1/hrs)	0.99 (-0.02-1.99)	0.43 (0.15-0.71)	**0.043**
K 2-1 (1/hrs)	1.26 (0.73-1.79)	0.73 (0.43-1.04)	**0.016**
V1 (dL/kg)	0.46 (0.28-0.65)	0.39 (0.33-0.45)	0.614
Secondary PK parameters			
Alpha (1/hrs)	2.80 (0.85-4.74)	1.29 (0.65-1.93)	**0.015**
Alpha HL (hrs)	0.71 (0.32-1.10)	2.18 (1.21-3.15)	0.051
Beta (1/hrs)	0.06 (0.04-0.07)	0.05 (0.04-0.06)	0.109
Beta HL (hrs)	13.07 (9.89-16.25)	18.23 (14.70-21.77)	0.086
Cl (dL/h/kg)	0.04 (0.02-0.07)	0.03 (0.02-0.04)	0.147
CLD2 (dL/h/kg)	0.37 (0.13-0.60)	0.12 (0.06-0.19)	**0.012**
C max (IU/dL)	77.31 (56.3-98.4)	105.99 (83.1-128.9)	0.055
*MRT (hrs)	18.38 (13.79-22.97)	23.40 (19.36-27.44)	0.134
V2 (dL/kg)	0.24 (0.10-0.38)	0.18 (0.15-0.22)	0.757
Vss (dL/kg)	0.69 (0.40-0.99)	0.57 (0.50-0.64)	0.467
*AUC (IU.h/dL)	994 (557-1430)	1467 (1188-1746)	0.058
AUCM (IU.h^2/dL)	21543 (8213-34872)	39157 (26667-51647)	0.057

The mean values and 95% CI are indicated. P, ANOVA of skewed variables logarithmically transformed; *not skewed variables. K 1-0, elimination rate from the central compartment; K 1-2, transfer rate from central (1) to peripheral (2) compartment; K 2-1, transfer rate from peripheral (2) to central (1) compartment; V1, Volume of central compartment. Alpha, alpha rate constant associated with the distribution phase; Alpha HL, alfa distribution half-life; Beta, beta rate constant associated with the elimination phase; Beta HL, beta elimination half-life; Cl, clearance; CLD2, inter-compartment clearance; C max, at zero time extrapolated FVIII concentration; MRT, mean residence time; V2, volume of peripheral compartment; Vss, volume of distribution at steady state; AUC, area under the curve; AUMC, the moment of AUC.

Table 2 Analysis of association between PK parameters and the *LDLR* c.1773C/T polymorphism

	***LDLR* c.1773C/T**			
	CC (n = 8)	CT (n = 20)	TT (n = 5)	P
Final PK parameters				
K 1-0 (1/hrs)	0.09 (0.05-0.12)	0.08 (0.06-0.10)	0.14 (-0.06-0.35)	0.646
K 1-2 (1/hrs)	0.26 (0.00-0.51)	0.54 (0.22-0.85)	1.41 (-1.05-3.87)	**0.043**
K 2-1 (1/hrs)	0.51 (0.15-0.86)	0.88 (0.56-1.21)	1.54 (0.30-2.78)	**0.034**
V1 (dL/kg)	0.43 (0.31-0.54)	0.37 (0.30-0.43)	0.56 (0.17-0.95)	0.769
Secondary PK parameters				
Alpha (1/hrs)	0.78 (0.17-1.39)	1.59 (0.89-2.30)	3.91 (-0.62-8.43)	**0.011**
Alpha HL (hrs)	2.51 (0.42-4.60)	1.76 (0.85-2.66)	0.42 (-0.03-0.86)	**0.019**
Beta (1/hrs)	0.05 (0.03-0.07)	0.05 (0.04-0.06)	0.06 (0.03-0.09)	0.646
Beta HL (hrs)	15.7 (10.6-20.7)	18.0 (14.0-22.0)	12.9 (7.21-18.56)	0.614
Cl (dL/h/kg)	0.04 (0.02-0.06)	0.03 (0.02-0.04)	0.06 (0.00-0.11)	0.509
CLD2 (dL/h/kg)	0.10 (-0.01-0.22)	0.16 (0.08-0.23)	0.50 (-0.02-1.02)	**0.016**
C max (IU/dL)	75.3 (56.3-94.2)	110.7 (84.7-136.7)	78.9 (40.4-117.5)	0.648
*MRT (hrs)	20.1 (14.0-26.3)	23.5 (19.0-28.0)	18.34 (9.92-26.77)	0.907
V2 (dL/kg)	0.19 (0.11-0.27)	0.19 (0.15-0.23)	0.27 (-0.04-0.59)	0.874
Vss (dL/kg)	0.63 (0.44-0.81)	0.55 (0.47-0.63)	0.82 (0.23-1.41)	0.406
*AUC (IU.h/dL)	1099 (537-1661)	1490 (1195-1785)	1016 (153-1879)	0.911
AUCM (IU.h^2/dL)	25818 (6777-44860)	39961 (26036-53886)	22054 (278-43830)	0.852

The mean values and 95% CI are indicated. P, ANOVA of skewed variables logarithmically transformed; *not skewed variables. K 1-0, elimination rate from the central compartment; K 1-2, transfer rate from central (1) to peripheral (2) compartment; K 2-1, transfer rate from peripheral (2) to central (1) compartment; V1, Volume of central compartment. Alpha, alpha rate constant associated with the distribution phase; Alpha HL, alfa distribution half-life; Beta, beta rate constant associated with the elimination phase; Beta HL, beta elimination half-life; Cl, clearance; CLD2, inter-compartment clearance; C max, at zero time extrapolated FVIII concentration; MRT, mean residence time; V2, volume of peripheral compartment; Vss, volume of distribution at steady state; AUC, area under the curve; AUMC, the moment of AUC.

Table 3 Linear regression model for predictors of the PK parameters variability

	ß-coefficient	P	
Final PK parameters			
K 1-2 (1/hrs)	-0.269	0.130	*ABO*
	0.276	0.119	*LDLR* c.1773C/T
K 2-1 (1/hrs)	-0.331	0.057	*ABO*
	0.273	0.113	*LDLR* c.1773C/T
Secondary PK parameters			
Alpha (1/hrs)	-0.311	0.065	*ABO*
	0.348	**0.040**	*LDLR* c.1773C/T
Alpha HL (hrs)	0.240	0.168	*ABO*
	-0.332	0.060	*LDLR* c.1773C/T
CLD2 (dL/h/kg)	-0.335	**0.049**	*ABO*
	0.318	0.060	*LDLR* c.1773C/T

K 1-2, transfer rate from central (1) to peripheral (2) compartment; K 2-1, transfer rate from peripheral (2) to central (1) compartment; Alpha, alpha rate constant associated with the distribution phase; Alpha HL, alfa distribution half-life; CLD2, inter-compartment clearance.

Fig. 1

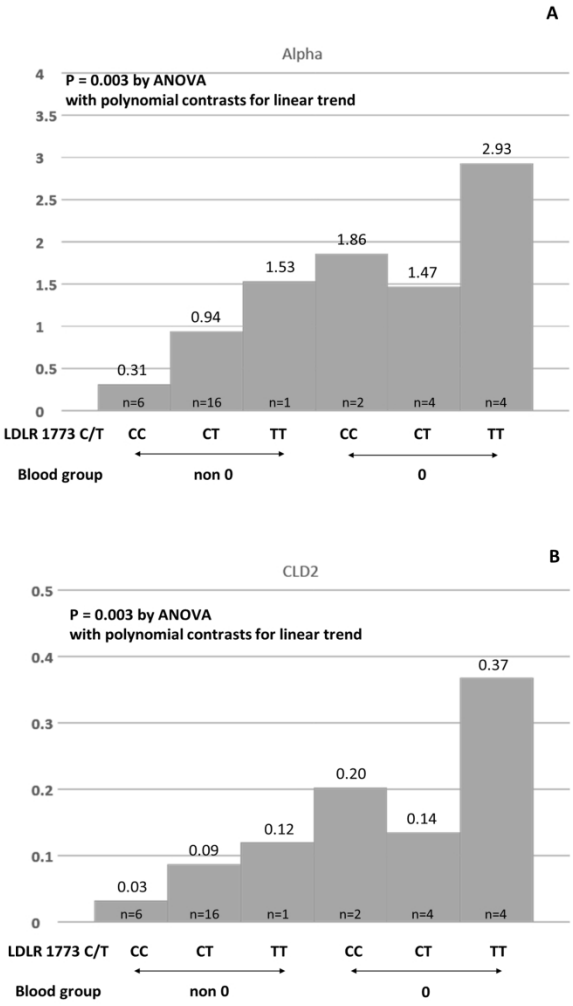

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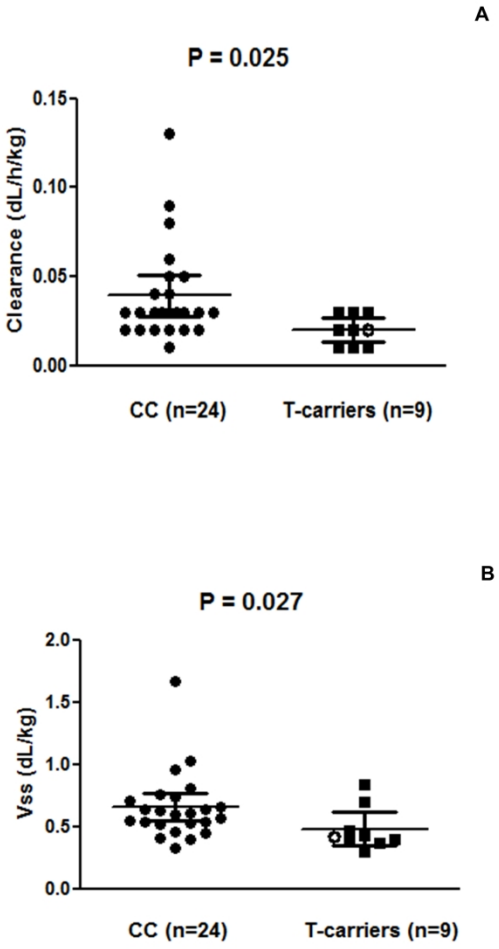
Fig. 2



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