In conclusion, we believe that MPV is a useful parameter for differentiating ITP from inherited macrothrombocytopenias, provided that appropriate cell counters are used. Nevertheless, a better standardization of the measurement of MPV is highly desirable and we are confident that reaching this goal will further increase the usefulness of platelet volume evaluation.

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A novel factor V mutation causes a normal activated protein C ratio despite the presence of a heterozygous *F5* R506Q (factor V Leiden) mutation

Activated protein C (APC) resistance (Dahlbäck *et al*, 1993) is a common risk factor for venous thromboembolism (Bucciarelli *et al*, 1999). This phenotype is highly associated with the *F5* R506Q (*F5* rs6025; factor V Leiden, FVL) mutation (Bertina *et al*, 1994). Heterozygous and homozygous discrepancies between *F5* R506Q genotype and phenotype (APC resistance), known as pseudo-homozygosity, have been described (Castaman *et al*, 1997; Brugge *et al*, 2005). This incidental phenomenon suggests that, based on APC-resistance testing, there is a homozygous *F5* R506Q mutation, yet, genetic testing only shows a heterozygous *F5* R506Q mutation (Simioni *et al*, 1996). In such cases, polymorphisms located on the second factor V (FV) protein coding allele (i.e. wild-type for *F5* R506Q) cause intracellular decreased FV protein production, leading to reduced FV activity (Castoldi *et al*, 1998). In contrast, the phenomenon "pseudo-wild-type" FVL, suggesting a wild-type *F5* R506Q according to APC resistance testing but heterozygous *F5* R506Q mutation by genetic testing, is infrequently described (Dargaud *et al*, 2003; Asselta *et al*, 2004). In these two studies, the polymorphisms responsible for the reduced FV protein levels were found to be located on the same FV protein coding allele, together with the *F5* R506Q mutation.

In this study, four cases with such a "pseudo-wild-type" FVL, based on a novel detected polymorphism, were investigated. An adolescent, healthy female subject was advised by her physician to determine her *F5* R506Q status before being prescribed hormonal contraceptives. She was a non-smoker with no personal or family history of any thromboembolic events. She was tested for both APC resistance and the *F5* R506Q mutation. Because there was a discrepancy between the functional APC resistance testing and the genetic *F5* R506Q testing results, her family was tested and a pedigree was established. (Supporting Information Data S1) In the index subject, a heterozygous *F5* R506Q mutation but a normal APC ratio were detected (Fig 1). The APC ratio was cross-checked using three different assays based on different FV activation pathways, which yielded consistent, normal results (Table I). When the *F5* gene was

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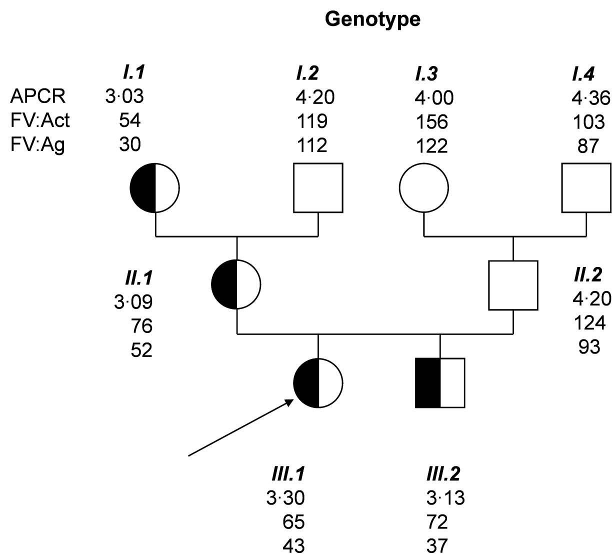

Fig 1. The INDEX subject is indicated by the arrow. Half-filled symbols denote carriers of the heterozygous *F5* R506Q (factor V Leiden) mutation and the novel heterozygous missense mutation *F5* G1718S (factor V Graz). Double heterozygosity for *F5* R506Q and *F5* G1718S is associated with a normal activated protein C ratio (APCR), low FV activity (FV:Act %) and low FV antigen (FV:Ag %). The mean FV:Act and FV:Ag values in the double heterozygous carriers for *F5* R506Q and *F5* G1718S were significantly lower than in wild-type carriers (67% vs. 125%, $P < 0{\cdot}005$ and 40% vs. 103%, $P < 0{\cdot}001$, respectively).

sequenced in the index subject with the heterozygous *F5* R506Q mutation, a novel heterozygous missense mutation 5326G>A (G1718S) in exon 16 of *F5* was detected (Fig S1). This novel mutation (*F5* G1718S) was registered as "factor V Graz" (FV Graz, GenBank accession number JF816043). Sequence analysis of *F5* G1718S in the PolyPhen-2 databank yielded in a "probably damaging" result [score 1·0] (Fig 2A-c, B). The FV activity (FV:Act) and the FV antigen (FV:Ag) levels of the index subject were significantly lower when compared to the mean values of the four non-FVL family members (65% vs. 125%, $P = 0{\cdot}023$; and 43% vs. 103%, $P = 0{\cdot}008$; respectively).

Her brother, mother, and maternal grandmother were found to be double heterozygous for *F5* R506Q and *F5* G1718S, and have a normal APC ratio. In these subjects the FV:Act and FV:Ag were also significantly lower when compared to those of the non-*F5* R506Q/non-*F5* G1718S family members (Fig 1).

Her father, paternal grandparents and maternal grandfather were each found to be wild-type for *F5* R506Q and *F5* G1718S, and have normal APC ratio, FV:Act and FV:Ag (Fig 1).

In the investigated family, three female subjects and one male subject showed a discrepancy between genotype and phenotype: a heterozygous *F5* R506Q mutation but normal APC ratios, which was caused by a novel sequenced mutation (*F5* G1718S). Interestingly, this novel *F5* G1718S mutation is located on the same allele as the heterozygous *F5* R506Q mutation. Therefore, we hypothesized that this new *F5* G1718S mutation is responsible for the low FV:Ag level. In this case, only a reduced amount of normal FV, which is coded by the second unaffected allele, is available and accounts for a wild-type-like normal APC ratio, suggesting a "pseudo-wild-type" for the *F5* R506Q mutation (Fig 2A-c).

This infrequent phenomenon (Dargaud *et al*, 2003; Asselta *et al*, 2004) was explained by a missense or a frameshift mutation on the *F5* gene, which causes amino acids to be exchanged or prematurely terminated, resulting in reduced FV protein levels. Published exchanges of amino acids in the region 1608 and 1745 of the *F5* gene result in low FV protein levels (Fig 2B) (Castoldi *et al*, 2000). Based on these findings, our novel missense mutation, detected at position 1718 of the FV protein, lies within the described region 1608 – 1745, and therefore seems to be the reason for both the markedly reduced FV:Act and FV:Ag levels measured in our four study subjects. When comparing our findings of the "pseudo-wild-type" FVL to the well-described pseudo-homozygous FVL, the main difference exists in the location of the *F5* R506Q mutation and the additional missense mutation on the *F5* gene causing the reduced FV protein levels: For "pseudo-wild-type" FVL, both mutations have to be located on the same FV protein coding allele; whereas, for pseudo-homozygous FVL, the mutations have to be located on the different FV protein coding alleles, as demonstrated (Fig 2A, Table S1).

The medical history of the entire family was ascertained and considered when evaluating the "pseudo wild-type" FVL test results and determining the haemostasis risk burden. None of the subjects had a history of thromboembolic events or any bleeding complications. Therefore, it can be assumed that the thromboembolic risk that is usually associated with a heterozygous *F5* R506Q mutation is no longer reliable if missense mutations, e.g., the *F5* G1718S mutation, located on the same allele as the *F5* R506Q mutation, are present.

In summary, the *F5* G1718S mutation causes a normal APC ratio despite the presence of a heterozygous *F5* R506Q mutation – described as a "pseudo-wild-type" FVL.

In these four cases, testing for only the *F5* R506Q genotype would have failed to determine the individual's thromboembolic risk associated with the heterozygous *F5* R506Q mutation. As numerous relevant missense mutations may exist on the *F5* gene that have not yet been detected or described, clinicians should be aware of this possible discrepancy between genetic (*F5* R506Q) and phenotypic (APC resistance) testing for FVL before drawing conclusions for thromboembolic prophylaxis and therapeutic management, especially when prescribing drugs such as combined oral contraceptives or hormonal replacement therapy.

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Table I. Activated protein C (APC) ratios in the plasma of all family members using three different test systems [Pefakit® APC Resistance (APC-R) Factor V Leiden assay, HEMOCLOT® Quanti V-L and COATEST® APC™ Resistance] in comparison with the *F5* genotypes [*F5* R506Q (FV Leiden) and *F5* G1718S (FV Graz)].

Family member	*F5* R506Q (Factor V Leiden)	*F5* G1718S (Factor V Graz)	Pefakit® [ratio]	HEMOCLOT® [%]	COATEST® [ratio]
Cut off			>2·9	<10	>2·2
I.1	HE	HE	3·03	7·95	2·34
I.2	WT	WT	4·20	3·15	2·87
I.3	WT	WT	4·00	2·15	2·83
I.4	WT	WT	4·36	2·30	2·50
II.1	HE	HE	3·09	7·35	2·39
II.2	WT	WT	4·20	2·95	3·00
INDEX III.1	HE	HE	3·30	6·63	2·42
III.2	HE	HE	3·13	7·45	2·52
Controls *F5* R506Q carriers	HE	WT	1·4–2·5	14–86	1·3–2·2

HE, heterozygous; WT, wild-type.

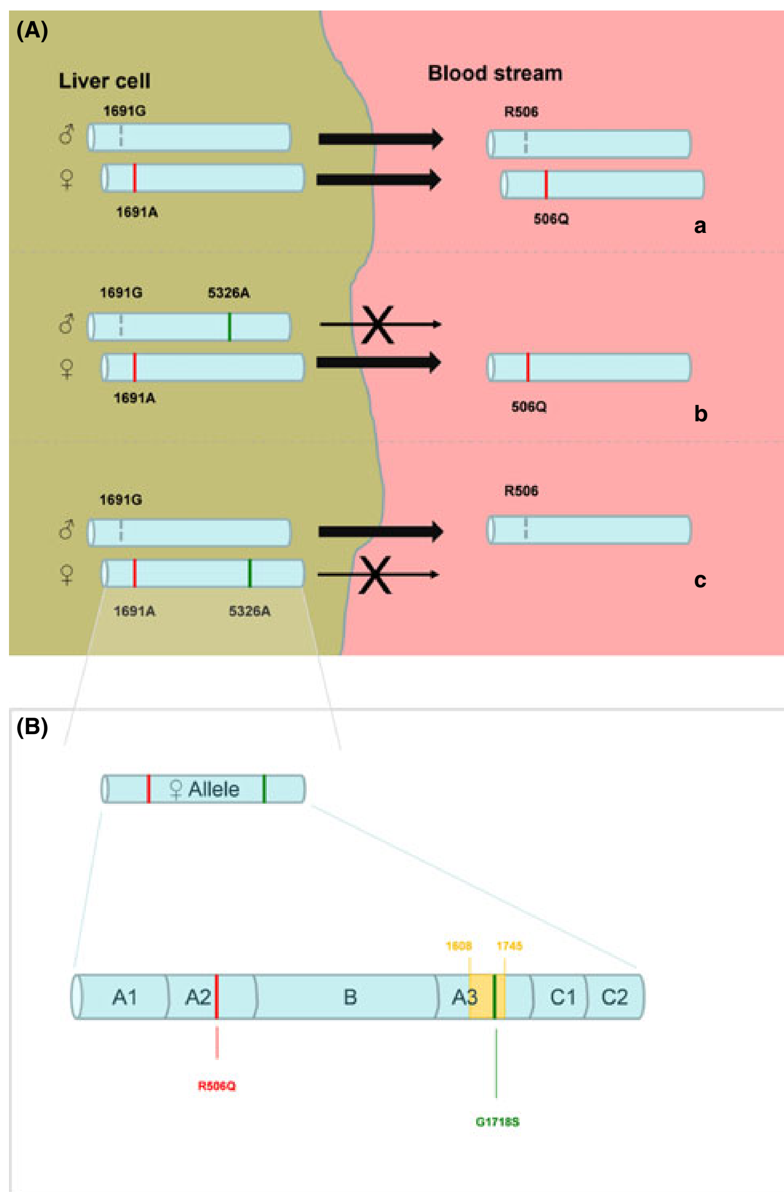

Fig 2. (A, B) Co-inheritance of heterozygous factor V Leiden mutation (*F5* 1691G>A, *F5* R506Q) and heterozygous factor V Graz (*F5* 5326G>A, *F5* G1718S): The factor V Graz mutation is located in the A3 domain of the coagulation factor V; Mutations between the amino acid positions 1608 to 1745 are responsible for a premature factor V protein degradation and lead to the expression of a reduced amount of factor V protein (B). (a) Inheritance of heterozygous *F5* R506Q mutation, leading to a normal amount of factor V protein; half of the protein is resistant to activated protein C (= APC resistance present). (b) The co-inheritance of *F5* R506Q mutation and the *F5* G17181S mutation on different alleles results in the expression of the FV Leiden protein only (= pseudo-homozygous APC resistance). (c) In the studied family, the co-inheritance of the *F5* G17181S mutation and *F5* R506Q mutation on the same allele leads to the expression of the non-FV Leiden protein only, resulting in a normal APC ratio (= "pseudo-wild-type" APC resistance).

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Author contributions

FP, BH study design. FP, EMM, WR, HK performed analysis. FP, RBR, HK wrote paper. KLS edited paper. MTW, HM, ECW, GS supported discussion.

Conflict of interest

None declared.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1. Methods and materials.

Fig S1. Detection of the novel mutation 5326G>A (G1718S) in exon 16 of the F5 gene - Sequencing protocol and RFLP analysis of the female index patient.

Table S1. Phenotypic results of different co-inherited factor V Leiden heterozygous conditions in the presence of the novel heterozygous factor V Graz polymorphism and the prevalence of these conditions.

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