Reference values of coagulation assays performed for thrombophilia screening after a first venous thrombosis and their intra-patient associations

Jean-Christophe Gris, Éva Cochery-Nouvellon, Chloé Bourguignon, Éric Mercier, Sylvie Bouvier, Isabelle Quéré, Antonia Perez-Martin, Nicolas Molinari, Éric Matzner-Lober

REFERENCE VALUES OF COAGULATION ASSAYS PERFORMED FOR THROMBOPHILIA SCREENING AFTER A FIRST VENOUS THROMBOSIS AND THEIR INTRA-PATIENT ASSOCIATIONS.

Jean-Christophe Gris[1-4], Éva Cochery-Nouvellon[1,3], Chloé Bourguignon[1,3], Éric Mercier[1-3], Sylvie Bouvier[1,3], Isabelle Quéré[3,5], Antonia Perez-Martin[3,6], Nicolas Molinari[2-3], Éric Matzner-Lober[3,7].

1 Department of Haematology, CHU Nîmes, Univ Montpellier, Nîmes, France

2 Faculty of Pharmaceutical and Biological Sciences, Montpellier University, France

3 UMR UA11 INSERM Institut Desbrest d'Epidémiologie et de Santé Publique - Montpellier University, France

4 I.M. Sechenov First Moscow State Medical University, Moscow, Russian Federation

5 Department of Vascular Medicine, University Hospital, Montpellier, France

6 Department of Vascular Medicine, CHU Nîmes, Univ Montpellier, Nîmes, France

7 CREST UMR 9194, ENSAE Formation Continue, 91120 Palaiseau, France

Corresponding author:

Jean-Christophe Gris, M.D., Ph.D.,

Département d'Hématologie, C.H.U.,

Groupe Hospitalo-Universitaire Caremeau, place du Pr. Robert Debré

F-30029 Nîmes cedex 9 France

e-mail : jean.christophe.gris@chu-nimes.fr

Tel : +33 466 683 211 fax: +33 466 683 648

ORCID: https://orcid.org/0000-0002-9899-9910

KEY WORDS

Thrombophilia, thrombosis, coagulation, references, laboratory score.

Word count: abstract 249, text 3,566

Tables: n=2; figures: n=4; supplementary table: n=1.

Highlights

- We lack reference values for thrombophilia screening after a first VTE
- We lack tools helping laboratory workers to validate results and their originality
- We describe the results of 12 coagulation parameters from 2,930 individual files
- We propose 2 individual scores testing similarity between the coagulation results

ABSTRACT

Introduction: No reference values are currently available for coagulation assays performed for thrombophilia screening prescribed according to guidelines, after a first venous thromboembolic (VTE) event, and we have no idea of the intra-patient associations between results.

Methods: We performed a retrospective study of consecutive prescriptions fulfilling guidelines in a French university hospital from 2010 to 2019 (n=3,842) from the Glims® laboratory information system. We collected results of 12 parameters: aPTT, PT, fibrinogen (Fg), one-stage clotting methods for factors VIII, IX, XI and II (FVIII, FIX, FXI, FII), antithrombin (using an amidolytic assay: AT), protein C and S (using clotting assays: PC and PS) and mixing tests of a lupus-anticoagulant sensitive aPTT and of DRVVT.

Results: We show the results of the 12 parameters from 3,603 individual files with less than 6 missing values, then describe these distributions and correlations between results from 2,930 files with no missing value. We give the frequency of results described as indicating a risk of first VTE or of VTE recurrence. We propose 2 quantitative scores linking the 12 parameters at the individual level and reflecting their degree of dispersion with respect to their mean, describe the values of these scores and their associations with thrombophilic results.

Conclusions: These normal values should help laboratory workers to validate process results and to assess their degree of originality. Our 2 scores should help to determine the intra-patient plausibility of associations of results. The usefulness of these laboratory scores for predicting clinically-relevant outcomes deserves to be investigated.

KEY WORDS

Thrombophilia, thrombosis, coagulation, references, laboratory score.

1. Introduction

In the 1990s and early 2000s, thrombophilia screening was commonly proposed to patients with venous thromboembolism (VTE) to assess underlying clotting tendencies. It is now less frequently prescribed to individuals with a personal or family history of VTE, although this is notion is controversial as thrombophilia testing has little clinical utility in most clinical situations [1]. The increasing professional consensus is that thrombophilia screening should be employed judiciously and only in patients for whom it is likely to alter clinical management [1, 2].

The coagulation laboratory is pivotal in thrombophilia screening, to consider the indications and refuting those contravening the current recommendations. It must also provide quality assurance, give results with indicative standards and references, and present the prescriber with an integrated overview of the coagulation status. To our knowledge, there is no published guideline for the results of coagulation tests usually prescribed for a thrombophilia assessment following a first VTE. Neither is there an integrated analysis of the relationship between the results of the various coagulation tests in that clinical situation. Therefore, except in rare cases with clear-cut diagnoses, it is difficult to interpret the degree of originality of the individual global coagulation test results, as well as their degree of plausibility of coexistence in a post-critical clinical situation. Thus, establishing relationships between individual sets of coagulation data and subsequent clinically-relevant outcomes is complex.

The aim of this study was to draft reference values for the most commonly tested parameters to determine coagulation status. We therefore performed a retrospective study to perform a descriptive exploratory analysis.

2. Methods

2.1 Patients

This is a retrospective study based on electronic data stored in the laboratory information system (Glims®, MIPS, Vincennes, France), limited to results generated by the coagulation sub-unit of the laboratory of Haematology, University Hospital (CHU) of Nîmes, France, between 1st January, 2010 and 1st January, 2019.

We extracted results corresponding to the pre-programed biological assessment entitled "thrombophilia assessment-coagulation", which only contains coagulation test results (solid-phase antiphospholipid antibodies and genetic studies performed in other sub-units of the Laboratory Medicine Department). We analysed prescriptions performed by any practitioner from the University Hospital of Nîmes, for which blood samples had been collected in the Outpatient Department of Haematology, located close to the laboratory of Haematology, thus guaranteeing optimal pre-analytical conditions.

We analysed the clinical reasons for the prescriptions from the institutional computerized patient medical record (Clinicom™, InterSytems, Lattes, France). Only those fulfilling all of the following conditions were retained, in line with our institutional guidelines on indications for laboratory analyses:

1- thrombophilia screening performed following a first deep vein thrombosis (DVT), with or without pulmonary embolism (PE), or after a first PE, all diagnosed under 60 years of age [3];

2- unprovoked DVT/PE, or DVT/PE not provoked by major transient risk factors during the 3 months before diagnosis [1,4], *i.e.* not associated with: major trauma, surgery with general anaesthesia for greater than 30 min., bedridden in hospital (only "bathroom privilege") for at least 3 days with an acute illness, Caesarean section. DVT/PE not provoked by the persistent risk factor active cancer [4].

3- thrombophilia screening performed at the end of anticoagulant therapy, when vitamin K antagonist treatment (VKA) had been stopped for at least 2 weeks, direct oral anticoagulant treatment (DOAC) for at least 5 days, whereby bridging was performed in patients with an unprovoked DVT/PE by a prophylactic-dose low-molecular weight heparin (LMWH) injected once a day around 8 p.m., with blood collected between 8 a.m. and 10 a.m. after fasting overnight. And in women in absence of any hormonal treatment.

2.2 Blood sampling and plasma preparation

Blood was drawn from an antecubital vein with a light tourniquet and a 21-G needle and collected into a tube containing 0.109 mol/L citrate (Becton Dickinson, Pont de Claix, France), at a 9:1 vol:vol ratio, after discarding the first few millilitres. Platelet-poor- plasma (PPP) was prepared by double centrifugation at 2,500 g for 15 minutes within 30 minutes following blood collection. Samples were aliquoted and stored (-80°C) in the Centre de Ressources Biologiques (NF S96-900, ISO 9001/ISO 20387 certified, number 210230/1285F), CHU Nîmes, until use within one week. Before use, frozen plasma samples were thawed by total immersion in a water bath at 37°C for 5 minutes, then gently homogenised.

2.3 Coagulation tests

All coagulation assays were performed using a STA-R Max automated coagulometer (Stago, Asnières, France) and the dedicated reagents, including calibrators, control plasmas and a reference normal plasma (normal human plasma pool: Pool Norm), according to the manufacturer's instructions.

A panel of 12 thombophilia assessment-coagulation assays were recorded. The following tests were performed: aPTT (PTT Automate reagent), PT (Néoplastine® CI Plus), procoagulant fibrinogen (Fg) using Clauss' method (Fibriprest®), factors II (FII) XI (FXI), IX (FIX) and VIII (FVIII) using one-stage clotting methods and deficient plasmas from Stago, antithrombin (AT) using an amidolytic assay (Stachrom ATIII®), protein C (PC) and protein S (PS) using clotting assays (Staclot® Protein C and Staclot® Protein S), lupus anticoagulant (LA) screening being performed with 2 reagents (PTT-LA®: an aPTT reagent sensitized to the detection of LA; and Staclot-DRVV®: a dilute Russel viper venom time DRVVT reagent).

Results of the aPTT and PT were given as the ratio between the patient's clotting time and the Pool Norm clotting time (patient:control ratio). Results of the PTT-LA and DRVVT were given as the ratio of the clotting times obtained with a 1:1 proportion of the patient's plasma and the Pool Norm (mixing test), and with the Pool Norm (mixing:control ratio).

The external quality control of the results of these 12 coagulation assays was performed by subscriptions to the ProBioCal® program, Lyon and to the Qualiris® progam, Stago, Asnières, France; and to the E.C.A.T. foundation program, Voorschoten, The

Netherlands. The Laboratory Medicine Department and the laboratory of Hematology of the university hospital of Nîmes are NF ISO 22870-2017 certified, number 8-3367.

The normal values suggested by the manufacturer of the reagents used for the 12 coagulation assays were checked on samples from 200 healthy blood donors, median age 50 years, interquartile rage (IQR) 20 years, range 16-60 years.

The GLIMS® codes used for retrieving all the tests' results were the following: aPTT ratio: HH_TCAPsurT; TQ ratio: HH_TQPsurT; Fg: HH_FG; FII: HH_F2; FXI: HH_F11; FIX: HH_F9; FVIII: HH_F8; AT: HH_AT3AM; PC: HH_PCF; PS: HH_PSF; PTT-LA, mixing:control ratio: HH_TCASMsurT; DRVVT, mixing:control ratio: HH_DRVTMsurT.

2.4 Statistical analysis

Qualitative data are described by their absolute values and frequency (%), quantitative values are described by their mean and standard deviation (SD) values and by values of key percentiles (0.5, 1, 2.5, 5, 10, 25, 33.3, 50, 66.6, 75, 90, 95, 97.5, 99, 99.5).
Matrix of correlation between quantitative data was computed using Pearson's correlation coefficients ρ. The Mann-Whitney-Wilcoxon rank sum test was used to compare 2 sets of quantitative data and the Kruskal-Wallis ANOVA by ranks to compare more than 2 sets of data. Cross-tabulation and chi-squared test were used for qualitative data.
We generated 2 scores aiming to estimate, at the individual level, the degree of similarity between the 12 coagulation parameters by reference to their mean values, and to identify files with a partial individual loss of similarity between the studied parameters. The 12 studied laboratory variables from each observation were scaled by dividing the centred variable by their standard deviations (difference between individual values and the corresponding means divided by the standard deviation of the variable).
The score number 1 was computing by the sum of the squared scaling values multiplied by the sign of the individual difference to the mean value of the corresponding variable.
The score number 2 was computing by the sum of the squared scaling values multiplied by the sign of the quantity reflected the thrombotic risk: for instance, values obtained with PC values lower than the mean gave positive entities and higher than the mean negative ones, but F8 values lower than the mean gave negative results and those higher than the mean positive results.

Finally, the values of the 12 entities computed for the 12 variables for each score were added and divided it by 12.

A didactic working example showing how to calculate score 1 and score 2 is developed in the supplementary Table 1.

$P<0.05$ was considered statistically significant.

3. Results

A total of 4,593 thrombophilia files were screened, of which 3,842 fulfilled all the inclusion criteria. The reasons for non-inclusion of 751 files included: not after a first VTE, n=112 ; VTE diagnosis after 60 years: n=199; VTE provoked by a major transient risk factor: n= 277; patient under any anticoagulant therapy: n=215; more than one of these reasons: n=52.

The characteristics of the corresponding included patients are described in Table 1:

Table 1. Patient characteristics (N= 3,842).

Quantitative variables: median, [lower quartile – upper quartile] (minimum-maximum) values; categorical variables: numbers (percentages). VTE: venous thromboembolism, PE: pulmonary embolism; DVT: deep vein thrombosis; BMI: body mass index

Age at thrombophilia screening	51 [37, 55] (18-60)
Male/Female	1,563 / 2,279 (40.7% / 59.3%)
BMI, $kg.m^{-2}$	25.7 [23.5, 28.8] (17.3 – 39.4)
VTE	
PE	1,451 (37.8%)
DVT	1,021 (26.6%)
Proximal	694 (18.1%)
Distal	327 (8.5%)
DVT and PE	1,370 (35.7%)
Provoking factors	

None	1,206 (31.4%)
Combined oral contraceptives	853 (22.2%; 37.4 % of the women)
Pregnancy	19 (0.5%; 0.8% of the women)
Puerperium	51 (1.3%; 2.2% of all the women)
Immobilisation	761 (19.8%)
Minor surgery	519 (13.5%)
Minor trauma	264 (6.9%)
Long-haul flight	39 (1%)
Localised infection	84 (2.2%)
Inflammatory disease flare-up	31 (0.8%)
Other	15 (0.4%)
Persistent risk factors	
Inflammatory disease	138 (3.6%)
High BMI, $\geq$ 25 kg.m^{-2}	1,641 (42.7%)
Overweight, < 30 kg.m^{-2}	954 (24.8%)
Obesity, < 35 kg.m^{-2}	517 (13.5%)
Class 1 obesity, < 40 kg.m^{-2}	170 (4.4%)

Among these 3,842 patients' files, the 12 laboratory parameters under focus were available in 2,930 cases and 912 files had at least one missing value. The incidence of missing values ranged from 15.5% (PC) to 4.2% (FVIII).

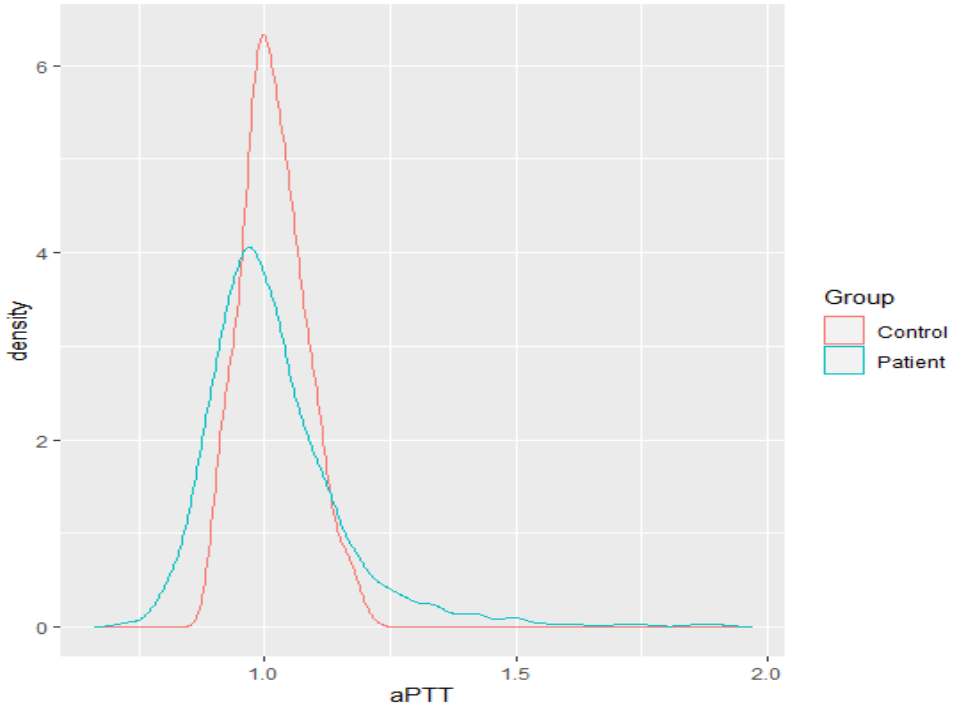
3.1 Files containing at least 50% of the data

We omitted files with at least 6 missing values, thus working on 3,603 individual files. The incidence of missing values was as follows: PC 11.85%, PS 11.71%, AT 8.91%, FII 7.35%, PTT-LA 3.69%, DRVVT 3.69%, FXI 2.58%, FIX 2.47%, FVIII 2.03%, Fg 1.83%, aPTT 1.17%, PT 0.99%.

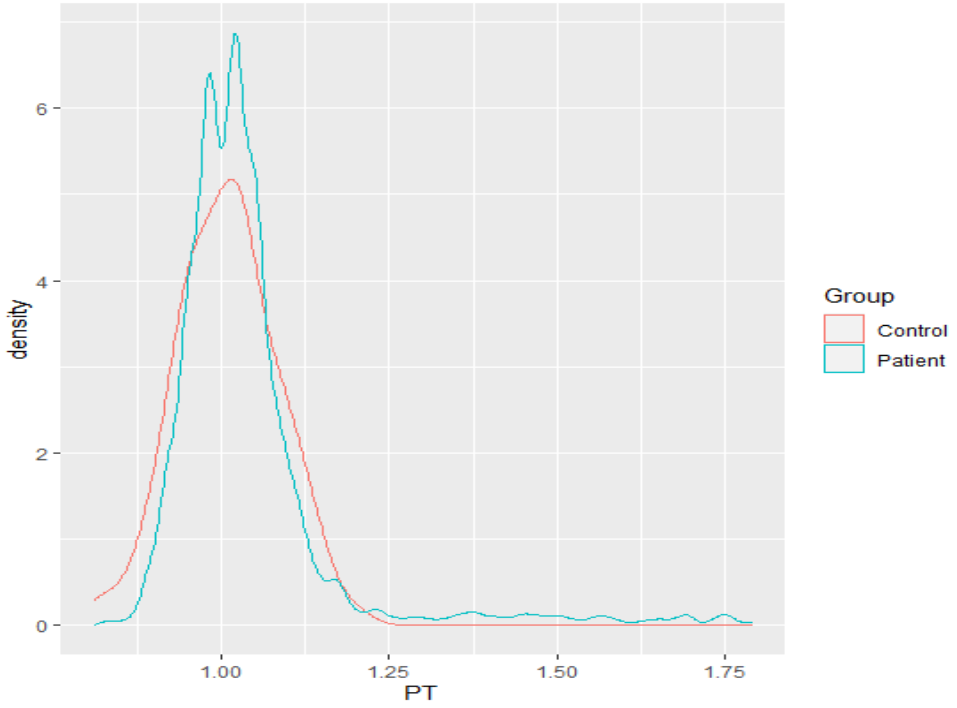
Figure 1 shows the distribution of the values of the 12 laboratory parameters, which can be compared to the distribution observed in healthy asymptomatic individuals (Fig. S1).

Fig. 1. Distribution of the values of the 12 tests in the 3,603 individual files with less than 6 missing values (blue lines) and in the 200 Controls (red lines) of **a.** aPTT, **b.** PT, **c.** FXI, **d.** FIX, **e.** FVIII, **f.** FII, **g.** Fg, **h.** AT, **i.** PC **j.** PS **k.** PTT-LA and **l.** DRVVT.
FXI, FIX, FVIII, FII, AT, PC and PS values are given as $IU.dL^{-1}$ or $U.dL^{-1}$, Fg values as $g.L^{-1}$; aPTT and PT as the patient:control ratios, PTT-LA and DRVVT as the mixing:control ratios.

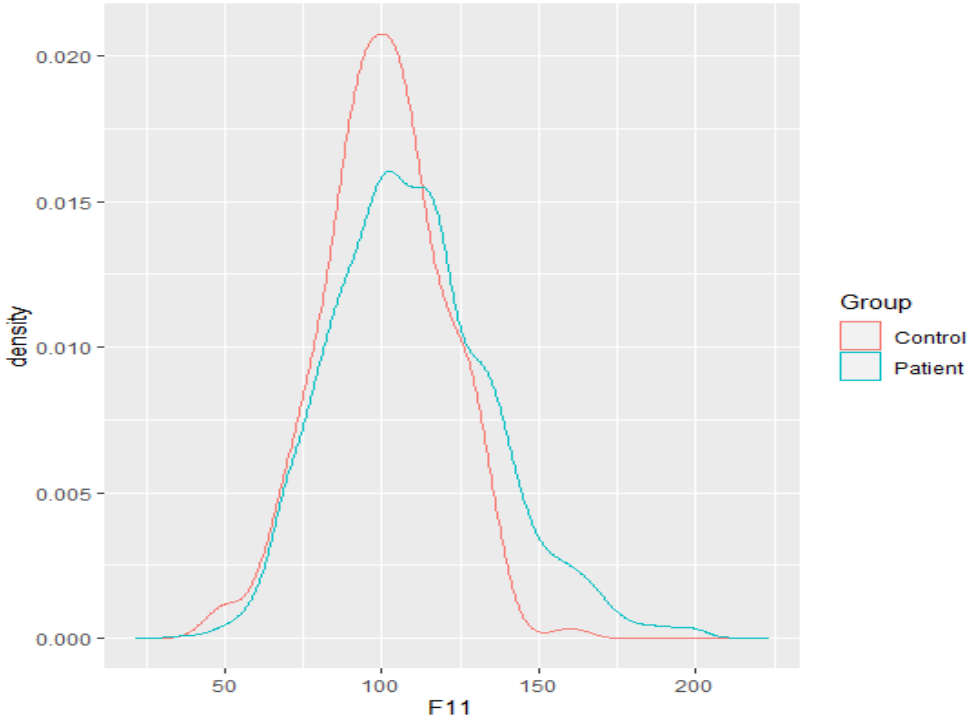
a Patients: N=3,561; Controls: N=200.



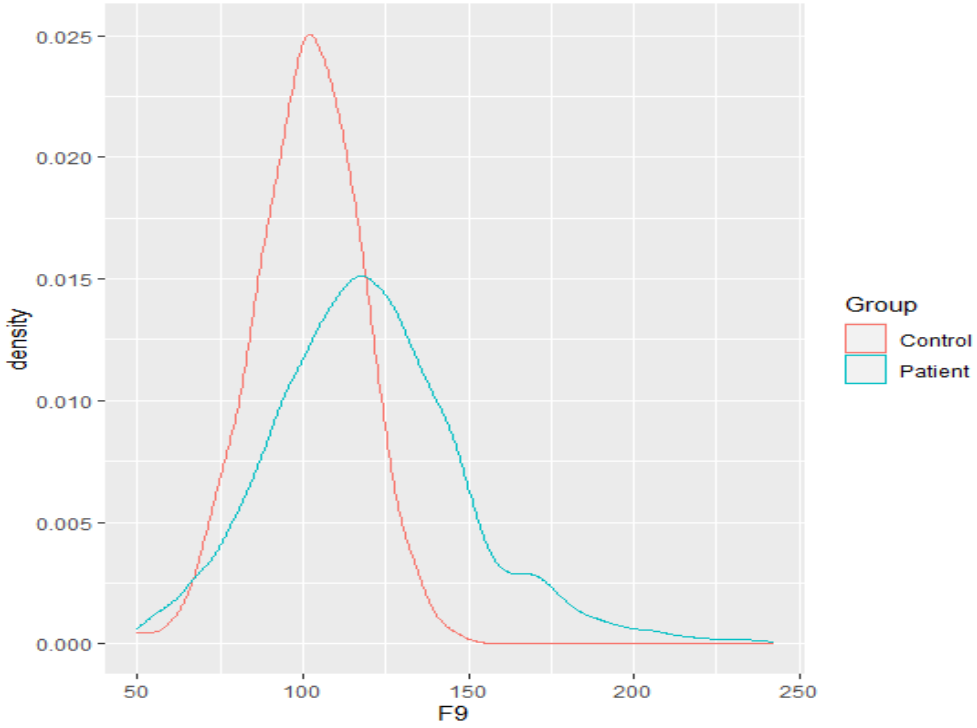
b Patients: N=3,567; Controls: N=200.



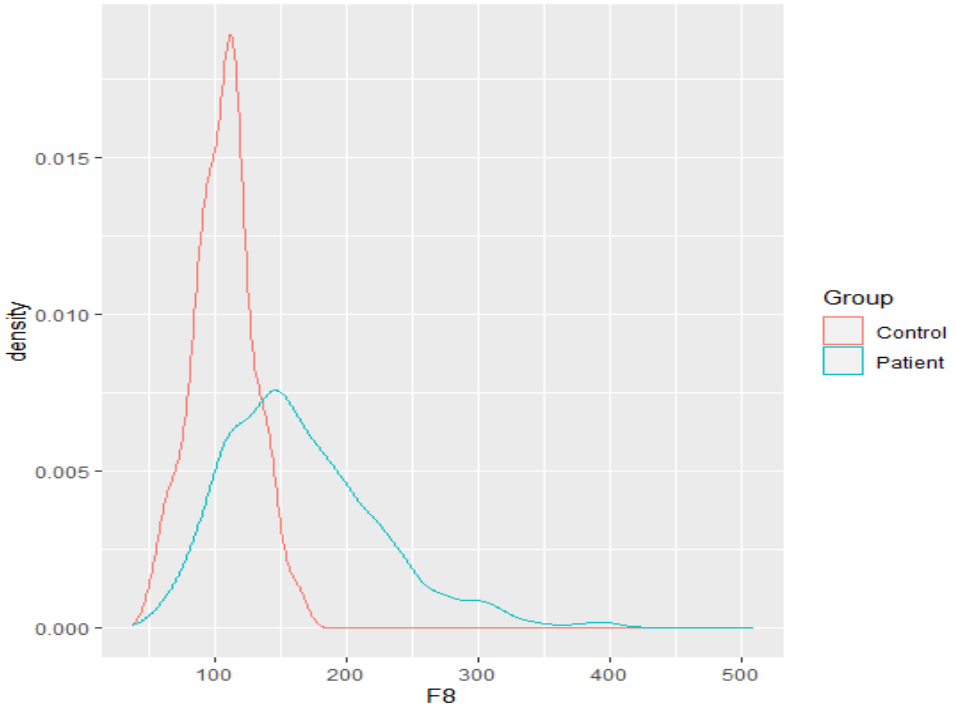
c Patients: N=3,510; Controls: N=200.



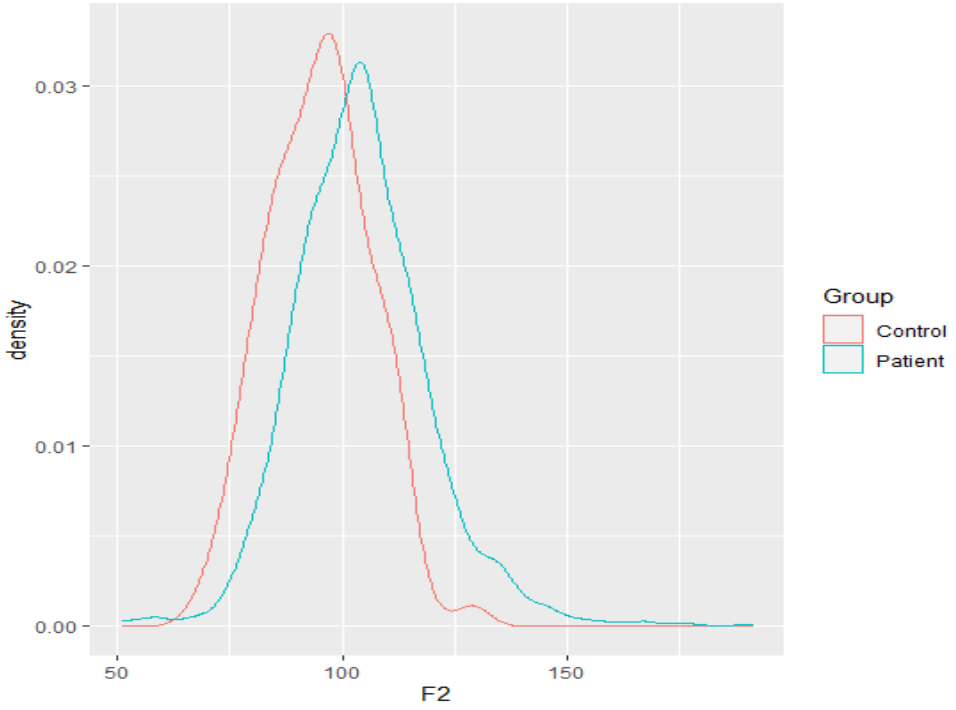
d Patients: N=3,514; Controls: N=200.



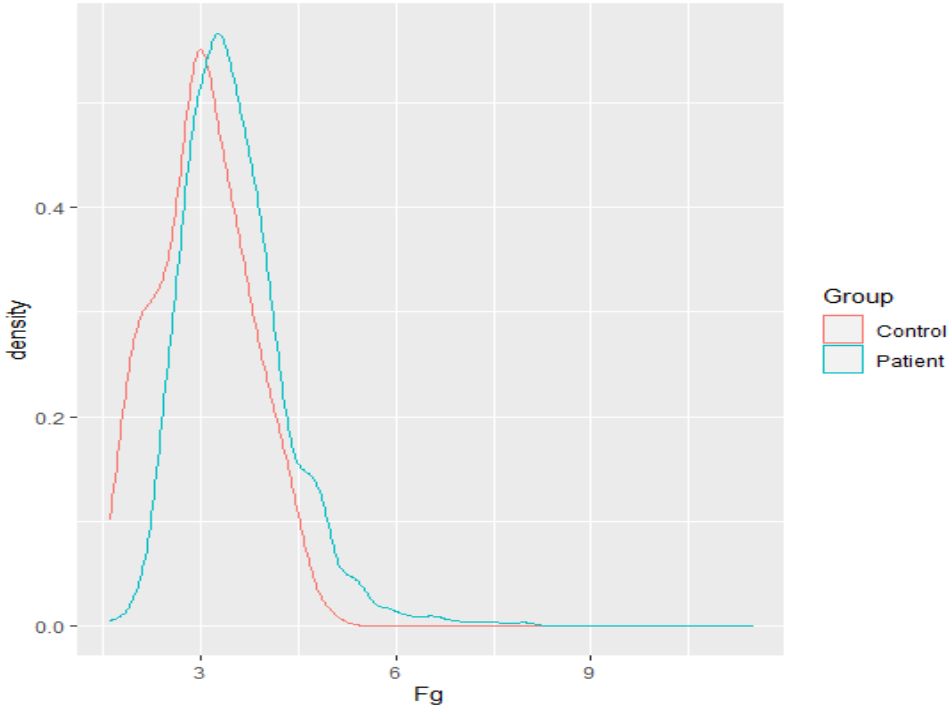
e Patients: N=3,530; Controls: N=200.



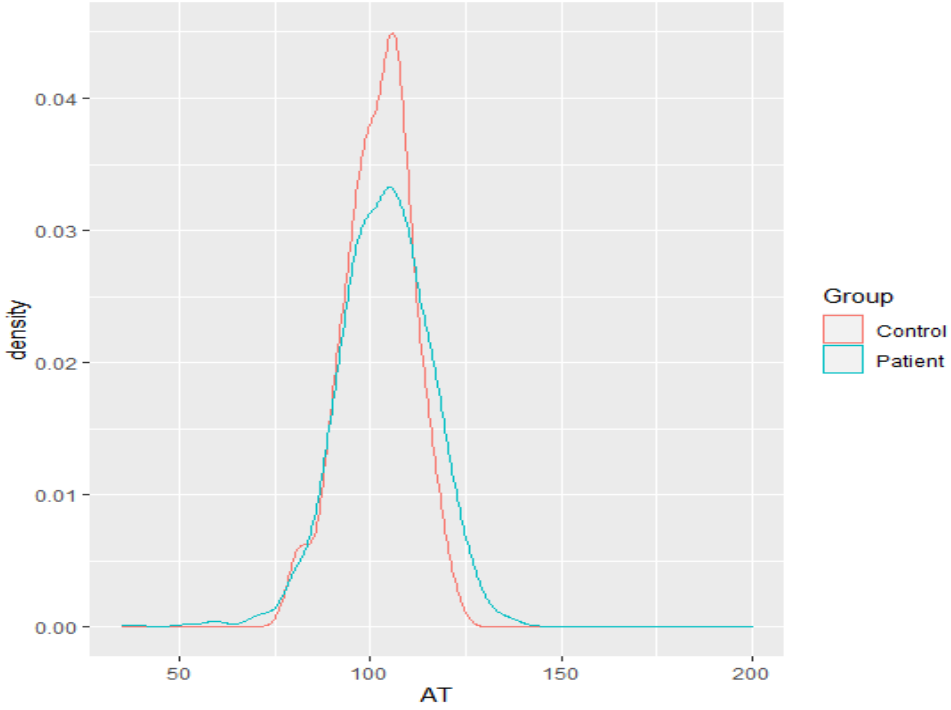
f Patients: N=3,338; Controls: N=200.



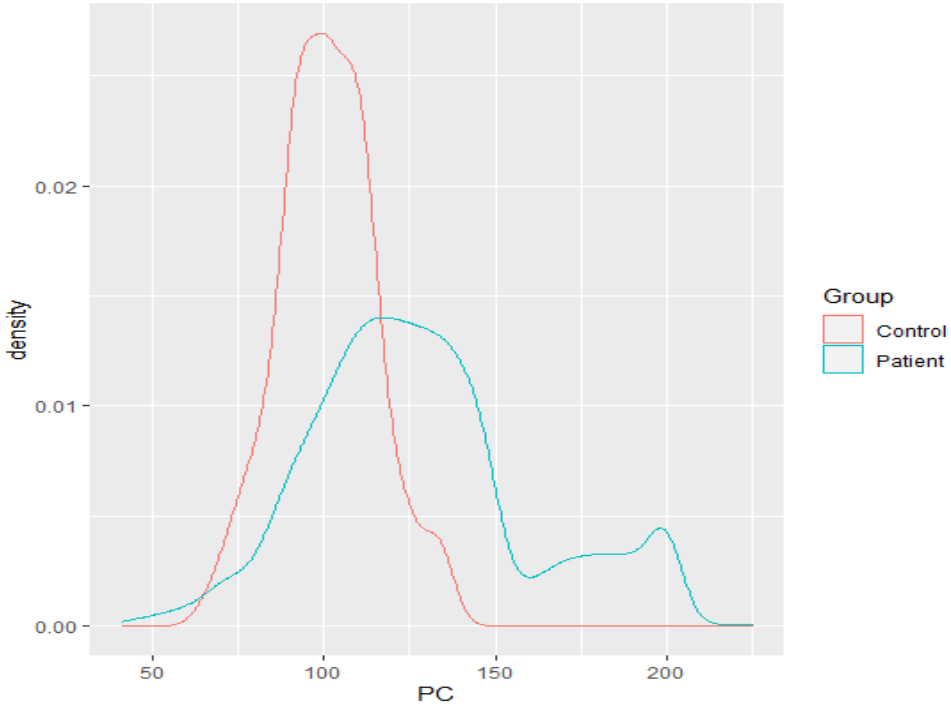
g Patients: N=3,537; Controls: N=200.



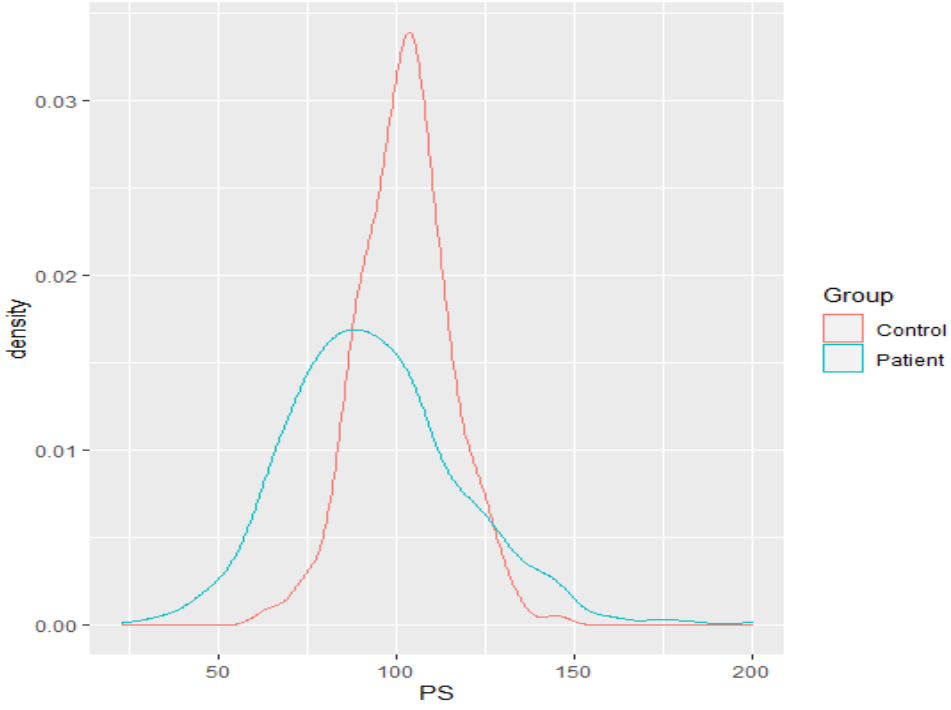
h Patients: N=3,282; Controls: N=200.



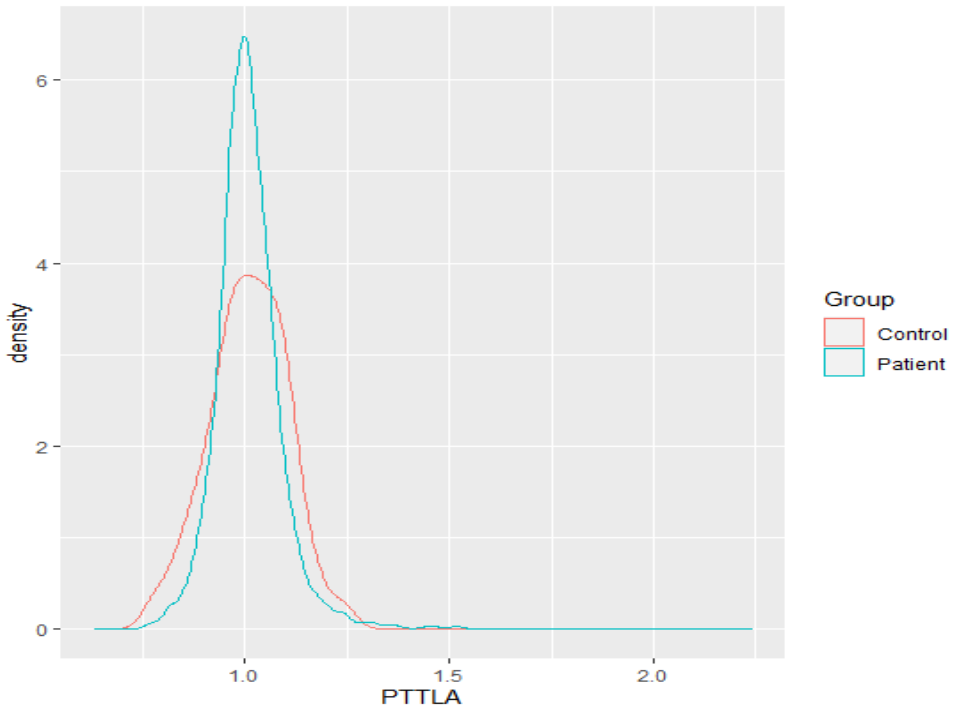
i Patients: N=3,176; Controls: N=200.



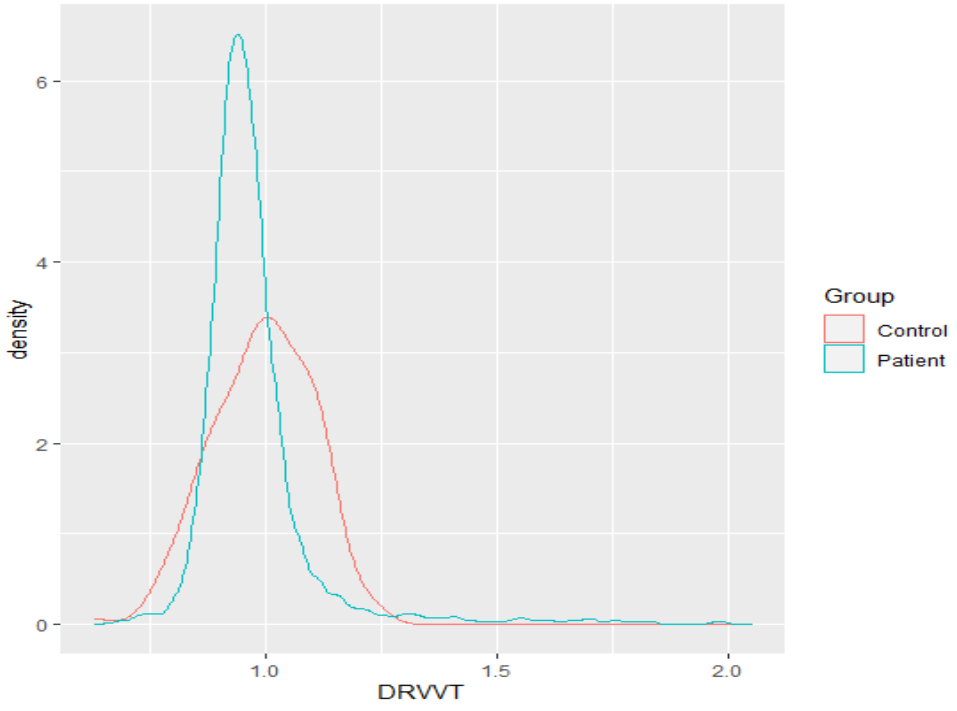
j Patients: N=3,181; Controls: N=200



k Patients: N=3,470; Controls: N=200



l Patients: N=3,470; Controls: N=200



One striking feature is the marked frequency of aPTT and PT patient: control ratios lower than 1.0 (Figure 1a-b). Also, the very wide distributions of FXI, FIX and FVIII values, with a predominance of high values and frequent appearances of shoulders on the right hand side of the curves (Figure 1c-e). FII and Fg distributions also share these features (Figure 1f-g). Interestingly, there is a clear existence of a marked subpopulation of high PC values (Figure 1h) and a marked incidence of PS values lower than 50 $IU.dL^{-1}$ coexisting with a shoulder of high values on the right of the distribution curve (Figure 1i).

3.2 Files with no missing data

Subsequently, we analysed the 2,930 files with no missing value. The values of the 12 laboratory parameters are detailed in Table 2.

Table 2. Description of the values of the 12 laboratory parameters in the 2,930 files with no missing value. FXI, FIX, FVIII, FII, AT, PC and PS values are given as $IU.dL^{-1}$ or $U.dL^{-1}$, Fg values as $g.L^{-1}$. P: percentile

	aPTT	PT	FXI	FIX	FVIII	FII	Fg	AT	PC	PS	PTT-LA	DRVVT
Mean	1.02	1.02	109.46	120.72	163.84	104.55	3.51	104.17	128.09	94.34	1.01	0.97
SD	0.17	0.10	25.53	27.13	58.13	14.50	0.80	12.11	32.33	24.58	0.10	0.14
P 0.5	0.78	0.88	55	65	58	72	2.01	68	52	36	0.81	0.74
P 1	0.79	0.89	61	67	65	75	2.11	74	61	43	0.82	0.78
P 2.5	0.82	0.91	67	74	76	79	2.31	81	72	52	0.86	0.83
P 5	0.85	0.92	71	80	86	83	2,43	86	82	59	0.89	0.85
P 10	0.88	0.94	78	88	99	88	2,62	90	91	66	0.92	0.88
P 25	0.93	0.98	91	102	122	95	2,96	96	107	77	0.96	0.91
P 33.3	0.95	0.98	97	108	135	98	3,12	99	113	83	0.97	0.92
P 50	0.99	1.01	108	119	155	104	3,40	104	125	92	1	0.95
P 66.6	1.03	1.04	118	130	180	109	3,73	109	137	102	1.03	0.98
P 75	1.06	1.05	125	136	196	113	3,93	112	143	109	1.05	0.99
P 90	1.16	1.10	142	154	238	122	4,56	119	180	127	1.10	1.06
P 95	1.26	1.14	155	170	272	131	4,91	123	197	138	1.14	1.16
P 97.5	1.39	1.23	166	181	301	137	5.34	126	200	146	1.20	1.40
P 99	1.59	1.48	179	199	338	146	5.89	131	200	162	1.33	1.71
P 99.5	1.86	1.68	192	212	389	154	6.50	135	200	175	1.47	1.81

The mean and median values of FXI, FIX, FVIII and PC are above 100 $U.dL^{-1}$, whereas mean PS is below 100 $U.dL^{-1}$. The matrix of correlations between the 12 parameters is shown in Table 3.

Table 3. Matrix of Pearson-type correlations between laboratory parameters in the 2,930 files with no missing value. Below the grey diagonal: coefficient of correlation; above: significance; *: p< .0001. NS: non-significant.

	aPTT	PT	FXI	FIX	FVIII	FII	Fg	AT	PC	PS	PTT-LA	DRVVT
aPTT		*	*	*	*	*	*	.0022	.1074 NS	.0006	*	*
PT	0.341		*	*	*	*	.0005	*	*	*	*	*
FXI	-0.323	-0.256		*	*	*	*	*	*	*	*	.0002
FIX	-0.305	-0.285	0.562		*	*	*	.0004	*	*	*	*
FVIII	-0.221	-0.100	0.428	0.565		*	*	.0053	*	*	*	.2627 NS
FII	-0.070	-0.305	0.307	0.295	0.100		*	*	*	*	.0005	.0086
Fg	0.087	-0.064	0.240	0.358	0.285	0.268		*	*	.0067	*	*
AT	-0.057	-0.149	0.158	0.065	-0.051	0.285	0.108		*	.0046	.0044	*
PC	0.030	-0.108	0.160	0.147	-0.070	0.353	0.089	0.287		*	.0800 NS	.0072
PS	0.064	0.105	0.111	0.105	-0.135	0.213	0.050	0.052	0.361		.0717 NS	*
PTT-LA	0.814	0.260	-0.256	-0.235	-0.216	-0.065	0.107	-0.053	-0.032	0.033		*
DRVVT	0.426	0.688	-0.070	-0.128	-0.021	-0.049	0.145	-0.091	0.050	0.276	0.442	

Highly significant correlations are seen, with strong correlations with coefficients higher than 0.5 observed between aPTT and PTT-LA, PT and DRVVT, FVIII and FIX, FIX and FXI.

From these data, we evaluated the incidence of results associated with the risk of VTE. aPTT ratios below 1.0 were found in 1,559 cases (46.8%): less than 0.87 [5] in 201 patients (6.86%) and less than 0.90 [6] in 408 patients (13.9%). aPTT ratios higher than 1.20, were observed in 218 cases (7.4%) with no evidence of any anticoagulant treatment interference. PT ratios lower than 1.0 were found in 1,159 cases (39.6%), with a minimum value of 0.83. PT ratios values over 1.20, were found in 80 patients (2.73%) with no evidence of any anticoagulant treatment interference.

Fg values higher than 5 g/L [7] were found in 121 patients (4.13%). Only 1 patient had a low fibrinogen concentration (< 1.50 g/L: 0.80 g/L), subsequently found to be related to a heterozygous Alès dysfibrinogenaemia [8].

High levels of coagulation factors of the intrinsic pathway have been associated with the risk of first VTE event [9-14] and with the risk of VTE recurrence after a first unprovoked VTE episode [15-19]. We found FVIII concentrations above 150 $IU.dL^{-1}$ [9] in 1,567 patients (53.5%) and above 234 $IU.dL^{-1}$ [15] in 321 patients (11.0%). Factor XI concentrations above 120.8 $IU.dL^{-1}$ and above 130.2 $IU.dL^{-1}$ [13] were found in 866 patients (29.6%) and 575 patients (19.6%), respectively, and above 150 $IU.dL^{-1}$ [18] in 194 patients (6.6%). Factor IX concentrations were above 129 $IU.dL^{-1}$ [14] in 1,022 patients (34.8 %), and above 138 $IU.dL^{-1}$ [19] in 692 patients (23.6%). We found high FII values above 115 $IU.dL^{-1}$ [20] in 566 cases (19.3%).

Focusing on physiological anticoagulants, 10 patients (0.34%) had circulating AT activities lower than 60 $IU.dL^{-1}$, all identified as constitutive deficiencies. A mild AT deficiency, lower than 80 $IU.dL^{-1}$ [21] was detected in 61 patients (2.08%), lower than 87 $IU.dL^{-1}$ in 170 (5.80%) patients and lower than 70 $IU.dL^{-1}$ [22] in 17 patients (0.58%). We detected a PC insufficiency with a residual clotting-based activity lower than 67 $IU.dL^{-1}$ [23] in 42 patients (1.43%), of whom 34 (1.16%) had levels lower than 65 $IU.dL^{-1}$, finally diagnosed with a constitutive deficiency. We found PS levels lower than 60 $IU.dL^{-1}$ in 162 patients (5.53%) but activities fell below 33 $IU.dL^{-1}$ [24] in only 8 patients (0.27%), all finally diagnosed with a PS deficiency.

Values of PTT-LA and DRVVT mixing tests exceeding 1.25 were respectively detected in 44 (1.50%) and 111 (3.79 %) of the patients. In patients with a high PTT-LA value, almost all had a high aPTT value (43/44), whereas nearly half of the patients with a high DRVVT value had a normal aPTT value (51/111), showing that a normal initial aPTT does not necessarily imply a normal DRVVT mixing test.

We studied the individual associations of the 6 coagulation factors with levels previously associated with the risk of VTE recurrence (FVIII > 234 $IU.dL^{-1}$, FIX > 138 $IU.dL^{-1}$, FXI > 150 $IU.dL^{-1}$, AT < 80 $IU.dL^{-1}$, PC < 67 $IU.dL^{-1}$ and PS < 33 $IU.dL^{-1}$). Fifty-five patients (1.88%) had elevated FVIII, FIX and FXI [25]: among whom, 2 had low AT values, and 1 had a low PC value. An additional 138 patients (4.70%) had both elevated FVIII and FIX: 5 had low AT values, 2 had low PC values and 1 a low PS value; and 16 patients (0.55%) had both elevated FVIII and FXI. Two patients with a low, deficient PC also had a low, non-deficient AT level.

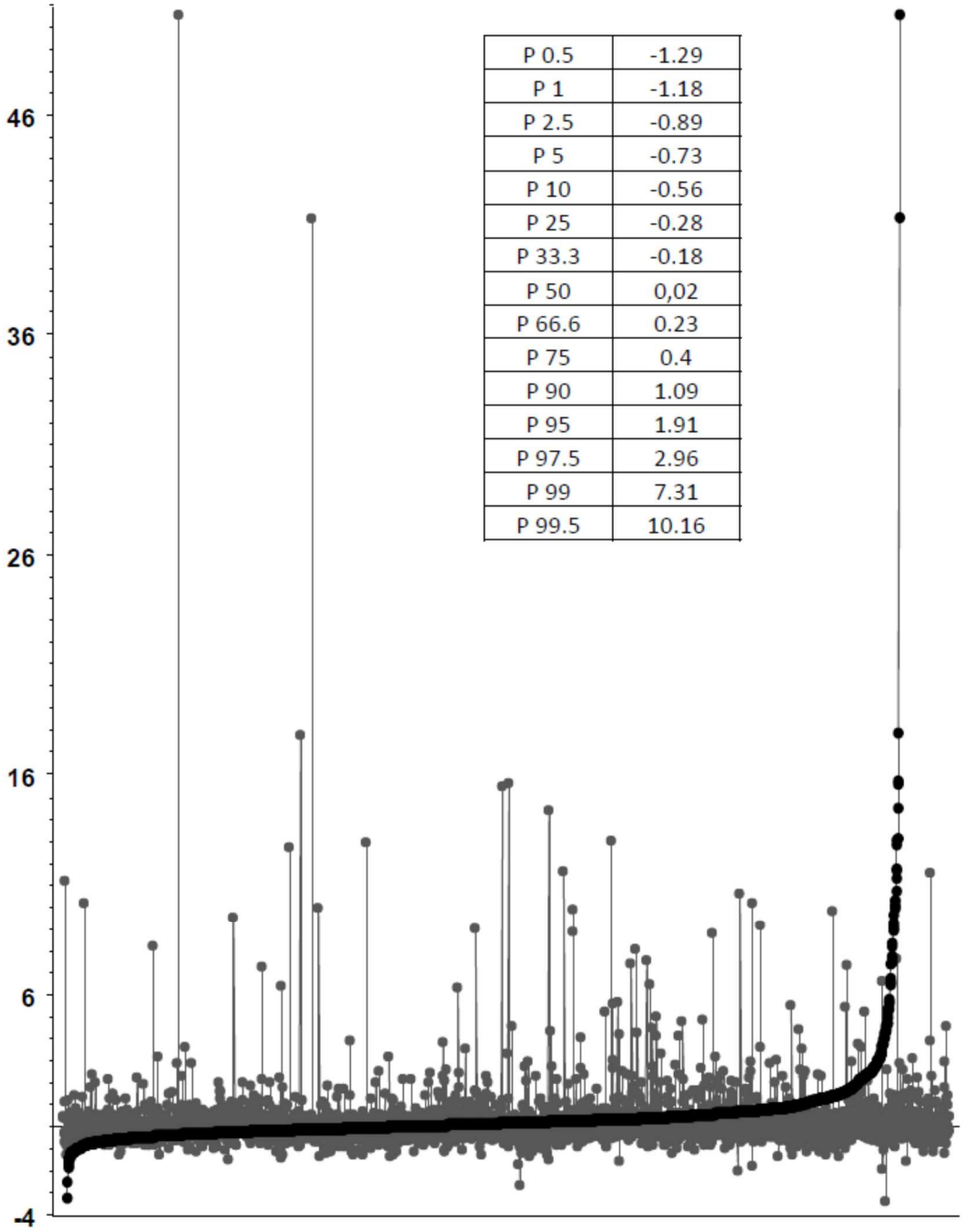
We generated 2 scores aiming to estimate, at the individual level, the degree of similarity between the 12 coagulation parameters by reference to their mean values, and to identify files with a partial individual loss of similarity between the studied parameters.

3.3 Scores estimating the individual degree of similarity between parameters

Finally, we studied the 2 scores, derived from files with no missing values.

Score 1, which reflects the individual degree of similarity between the 12 coagulation parameters by reference to their mean values, is described in Figure 2:

Fig. 2. Individual values of score number 1. P: percentile; grey dots and lines: individual data in the 2,930 files; black dots and lines: the same in increasing value order.

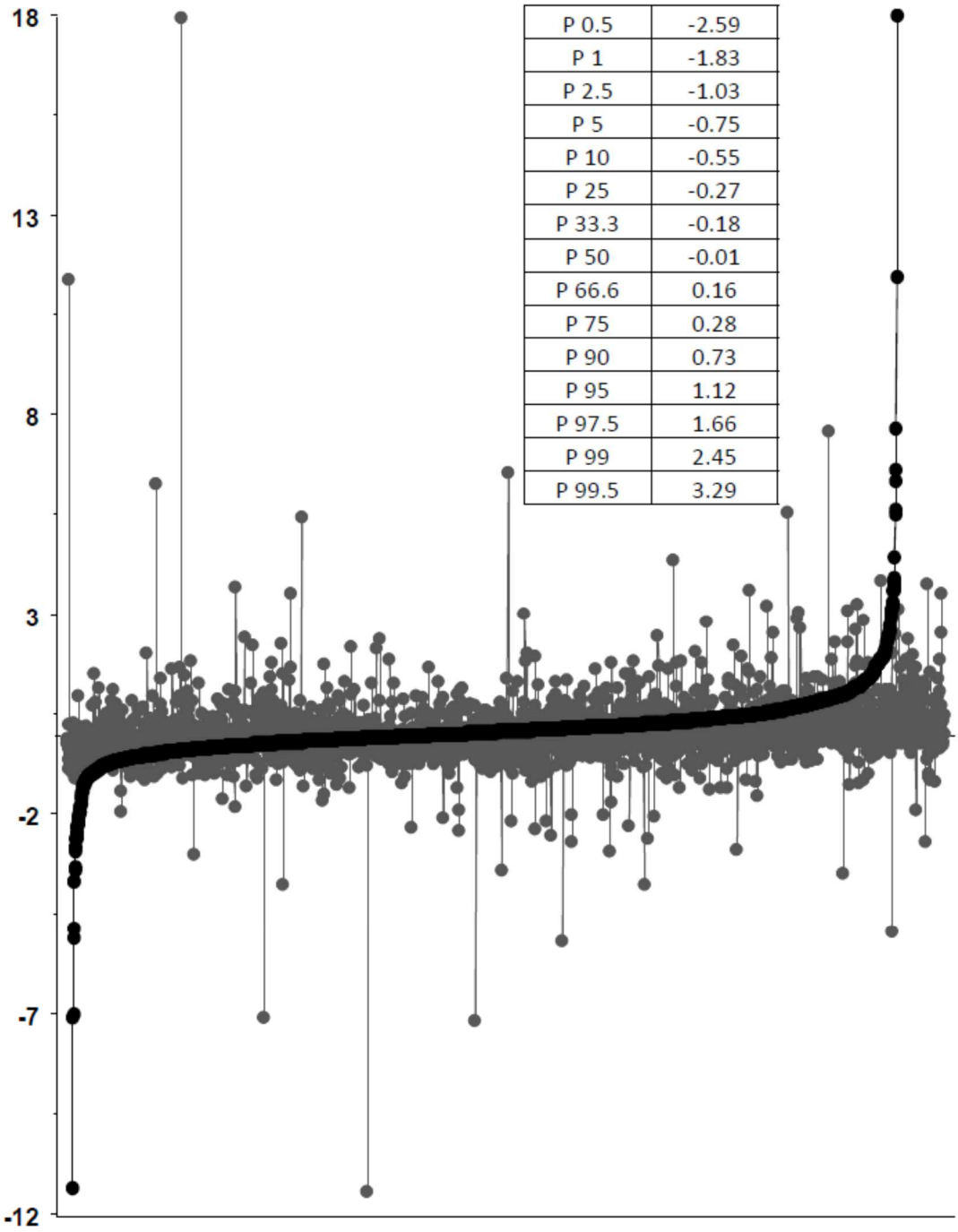


P 0.5	-1.29
P 1	-1.18
P 2.5	-0.89
P 5	-0.73
P 10	-0.56
P 25	-0.28
P 33.3	-0.18
P 50	0,02
P 66.6	0.23
P 75	0.4
P 90	1.09
P 95	1.91
P 97.5	2.96
P 99	7.31
P 99.5	10.16

The score 1 is calculated by taking the sum of 12 components and then dividing it by 12. Each component relates to one of the 12 biological parameters explored.
A given component, for a given patient, is calculated by first making the difference between the individual value of the biological parameter to which it relates, and the average of the values of this parameter observed in the 2,930 files without missing value. Then this difference is divided by the value of the standard deviation of the parameter calculated from the 2,930 records. Eventually, the obtained value is squared.

Score 2, change of score 1 more closely replicating the thrombotic risk, is described in Figure 3:

Fig. 3. Individual values of score number 2. P: percentile; grey dots and lines: individual data in the 2,930 files; black dots and lines: the same in increasing value order.



P 0.5	-2.59
P 1	-1.83
P 2.5	-1.03
P 5	-0.75
P 10	-0.55
P 25	-0.27
P 33.3	-0.18
P 50	-0.01
P 66.6	0.16
P 75	0.28
P 90	0.73
P 95	1.12
P 97.5	1.66
P 99	2.45
P 99.5	3.29

Score 2 uses the same 12 components as score 1, but the signs are adjusted.
If, for a given parameter, higher values are more at risk of thrombosis (FVIII, FXI ...), a positive difference between the individual value and the average of the values in the 2,930 records will generate a positive sign (a negative one if the difference is negative). Conversely, for a biological parameter whose lower values are more at risk of thrombosis (aPTT, AT, PC,…), a positive difference will generate a negative sign, and a negative difference a positive sign.

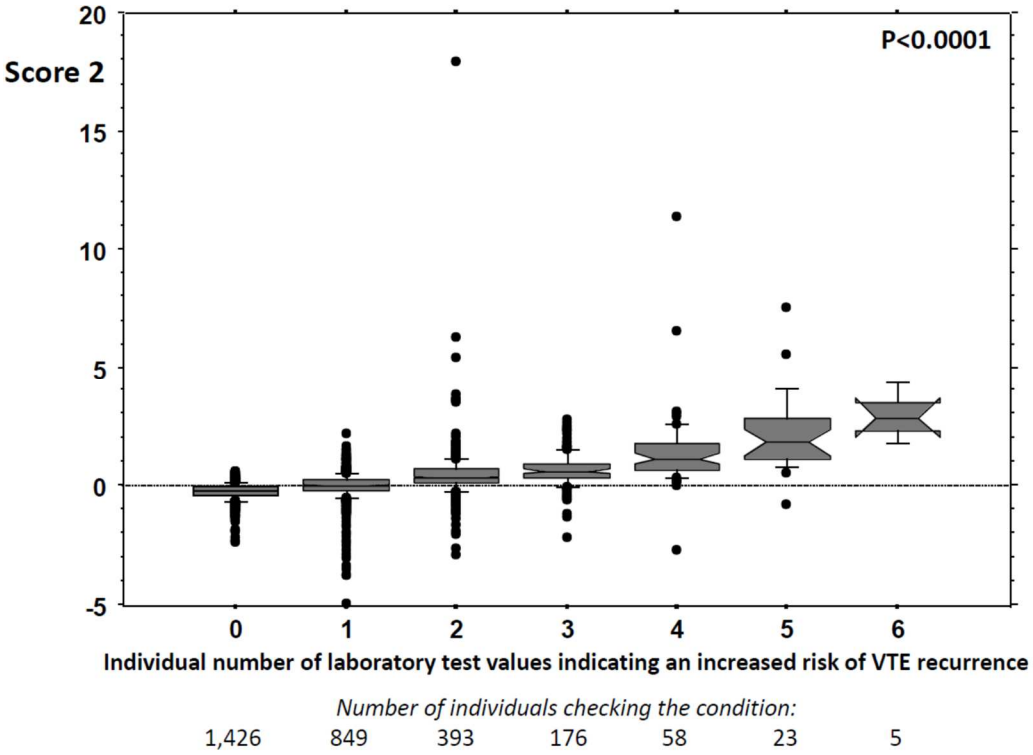
The distribution of the scores shows a high frequency of central values but also points deviating very notably from the median, with values above the median more frequent with score 1 and the values below the median more frequent with score 2.

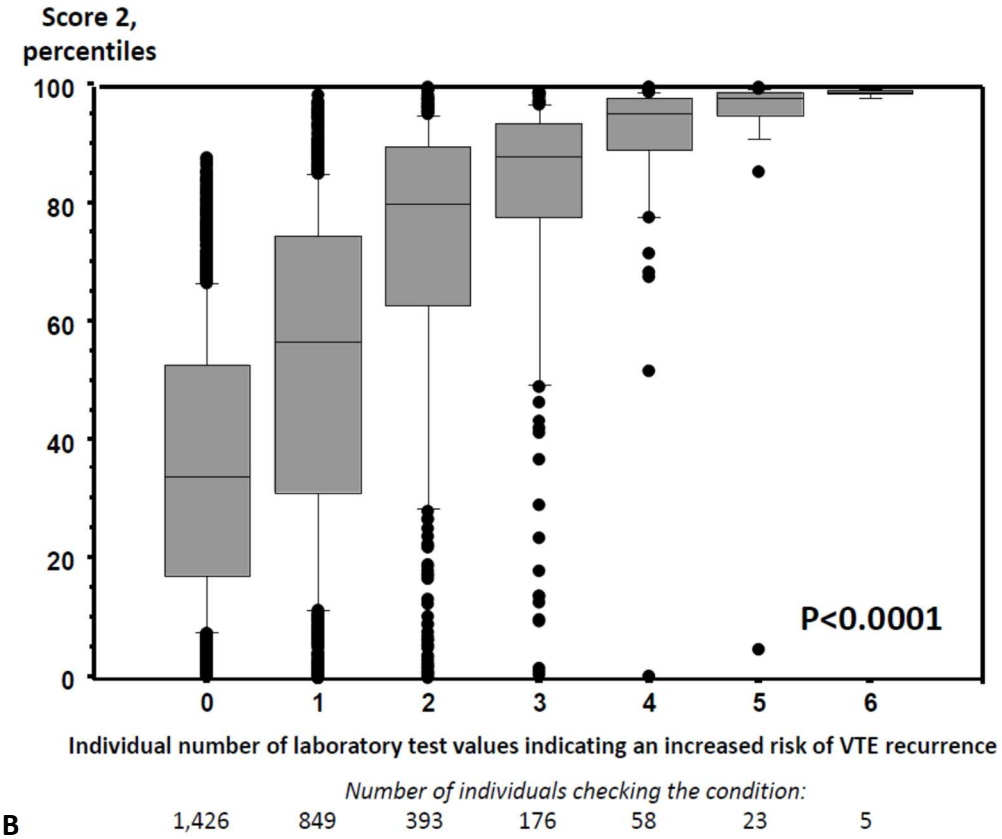
Patients showing high activity for at least one of FVIII, FXI, FXI, FII or Fg systematically had higher score 1 and score 2 values ($p<0.0001$) than those without these conditions. Files with low AT, PC and PS values had lower score 1 values ($p<0.0001$) but higher score 2 values ($p<0.0001$). Files with high PTT-LA and files with high DRVVT values had higher score 1 values ($p<0.0001$) but score 2 values were not statistically different (PTT-LA: $p=0.13$; DRVVT: $p=0.18$).

We next quantified the individual number of laboratory results associated with the risk of VTE recurrence (among the following: aPTT < 0.90 [6], FXI > 150 $IU.dL^{-1}$ [18], FIX > 138 $IU.dL^{-1}$ [19], FVIII > 234 $IU.dL^{-1}$ [15], FII > 115 $IU.dL^{-1}$ [20], Fg > 5 $g.L^{-1}$ [7], AT < 80 $IU.dL^{-1}$ [21], PC < 67 $IU.dL^{-1}$ [23], PS < 33 $IU.dL^{-1}$ [24], PTT-LA > 1.25, DRVVT > 1.25, the patients with between 0 to 6 of these results) and described the score 2 values, given as absolute values and as percentile values. We observed that a greater number of results indicating a clinical risk resulted in a higher score 2 ($p<0.0001$) (Figure 4).

Fig. 4. Values of the score 2 according to the individual number of laboratory results indicating an increased risk of VTE recurrence (aPTT < 0.90 [6], FXI > 150 IU.d^{L-1} [18], FIX > 138 IU.d^{L-1} [19], FVIII > 234 IU.d^{L-1} [15], FII > 115 IU.d^{L-1} [20], Fg > 5 g.L^{-1} [7], AT < 80 IU.d^{L-1} [21], PC < 67 IU.d^{L-1} [23], PS < 33 IU.d^{L-1} [24], PTT-LA > 1.25, DRVVT > 1.25). **A**: absolute values; **B**: percentile values.

A





B

4. Discussion

Published reference studies have analysed the association between coagulation factors and VTE, in terms of their ability to predict a first event or recurrence [5-24]. However, to our knowledge, no extensive reference values of coagulation assays performed in the context of thrombophilia screening are currently available. This lack of knowledge limits the ability to interpret the laboratory results. Our analysis retrospectively interpreted patient files showing evidence of a thrombophilia screening prescription performed according to guidelines, after a first VTE event, in absence of any anticoagulant treatment, and limited to procoagulant assays. We wished to propose a tool (score 1 and score 2) going beyond evaluation of the results compared to a population norm in relevant clinical conditions, to evaluate the individual link between the targeted 12 coagulation parameters. This tool would allow clarification of the plausibility and admissibility of the coexistence of results in the same patient.

The observed distributions of factor values differed markedly from those seen in a normal asymptomatic control population. The distributions were wider, more irregular and less symmetrical, often with a shoulder on the right, rarely centred on the value 100 U/dL^{-1}, showed a shift to the right and showed high values of variable intensity. Therefore, after a first VTE event, the circulating concentrations of coagulation factors deviates from that observed in an asymptomatic control population. An impact of subpopulations of patients, each having their own regular homogeneous distribution, the final distribution reflecting the summation of the effect of each of the subpopulations, can be suspected. For example, the second protein C peak, comprising high values centred on 200 $IU.dL^{-1}$, immediately suggests a subgroup, yet review of the files did not identify a common clinical or biological characteristic to categorise this population. In the post-critical situation when thrombophilia screenings are performed, the influence of acquired individual particularities (e.g. inflammatory level, the individual fat mass and the ongoing coagulation activation profile) on these distributions should be systematically investigated. Some extremely high FVIII activities have recently been described to arise from partial *F8* gene duplication [26], and it is

possible that some other constitutive traits will be described to explain other high circulating concentrations of coagulation factors, but they are likely to be rare.

The frequency of thrombophilic features we observed is in line with that usually described in Western countries and observed in case-control studies [5-24]. There is however, to our knowledge, no large scale study based on consecutive patients fulfilling the current recommendations for thrombophilia screening, and systematically including coagulation test-based detection of inherited thrombophilia defects, high levels of coagulation factors, and of a lupus anticoagulant activity through results of dedicated mixing tests. The most frequent findings were high levels of coagulation factors (FVIII, FXI, FIX and FII). A large amount of patients showed high levels of more than one coagulation factor exceeding the threshold for clinical risk of VTE recurrence. Some patients had simultaneously high FVIII, FIX and FXI levels. The secondary clinical prognosis of these patients is currently poorly documented. A recent large Dutch study on the relationship between various coagulation factor levels and VTE risk found FVIII (and its transporter von Willebrand factor (VWF)) to be associated with the highest VTE risk, the risks for other procoagulant factor levels being largely explained by FVIII and VWF [27]. The same group, studying the risk of VTE in patients over 70, found higher FVIII, FIX and FXI to be positively and independently associated with the risk of VTE, with high population attributable risks [28].

An original concept of our work was to evaluate, at the individual level, the degree of similarity between the 12 coagulation parameters by reference to their mean values, aiming to identify patients with a partial loss of concordance between the studied parameters. Normal values of coagulation tests are normally inferred from the statistical interpretation of distributions in a normal population. There is therefore likely that the abnormal thresholds in a thrombotic population do not correspond to those described in healthy controls, due to VTE-induced superimposed regulation processes of protein concentrations. The 2 scores that we propose aim to overcome the normal/abnormal result verdict by considering only the results from the same patient. It should help laboratory workers to evaluate the plausibility and probability of their results, providing them with a tool to interpret the global results, and to identify the abnormal parameters. The goal is also to identify the populations of individuals characterized by such a loss of homogeneity in their

coagulation results, which can be graduated since the scores are quantitative. The clinical significance of this must be evaluated in future studies.

Our work has some limitations. It is a retrospective, monocentric study. The reproducibility of results over time in patients has not been studied. Furthermore, only one commercial brand of reagents has been used and only coagulation tests were studied: the strict generalisability of our study data to other laboratory reagents is not warranted and needs specific developments. This is a first, purely laboratory work with no clinically-relevant endpoint. Finally, we did not include the analysis of clinical and biological covariates likely to modulate the laboratory results, and do not propose algorithms to interpret results according to these covariates.

It also has some strengths. We were able to collect a large number of cases, investigated according to guidelines, with a constancy of the pre-analytical and analytical steps. The data provide an overall description of the subject. It offers a new integrated technical approach to evaluate thrombophilia assessment. It opens up prospects for clinical extensions, and for VTE recurrence risk prediction models which integrate variable laboratory markers that are currently under investigation and validation [29].

Finally, our work establishes reference values of coagulation assays performed for thrombophilia screening prescribed according to guidelines and suggests new individual scores that can help biologists validate their results and assess their originality. We believe that our results can support the technical exercise of laboratory medicine in the field of coagulation. The clinical promises of our approach remain to be explored.

Data sharing statement

The dataset supporting the conclusions of this article are available in the clinical data repository of the University Hospital of Nîmes, Place du Pr. Robert Debré, 30029 Nîmes cedex 9, France, and by directly contacting the corresponding author (jean.christophe.gris@chu-nimes.fr).

Acknowledgements

We thank the members of the university hospital of Nîmes whose clinical activity fed the laboratory data herein studied; and particularly, by alphabetic order: Dr. Isabelle Aïchoun, Dr. Éric Arnaud , Dr. Jean-Pierre Balducchi, Dr. Jonathan Broner, Prof. Guillaume Cayla, Dr. Julien Frandon, Dr. Radjiv Goulabchand, Dr. Jérémy Laurent, Dr. Lorris Le Collen, Dr. Géraldine Lissade-Lavigne.

We also thank the staff at the "*Direction de la Recherche Clinique et de l'Innovation*", university hospital of Nîmes: S. Clément, C. Meyzonnier, N. Best, A. Megzari, E. Dupeyron, S. Granier, B. Lafont, C. Masseguin, S. de Boüard, L. Gazel and A.C. Simonet, and the staff of the "*Département de Biostatistique, Epidémiologie, Santé Publique et Information Médicale*": T. Mura, P. Fabbro-Peray.

We also thank Sarah Kabani and Teresa Sawyers for expert editorial assistance.

Funding

This work was conducted without any specific funding.

Conflicts of interest

The authors have no competing conflict of interest to declare.

Dr. Gris received research grants in the past from Bayer Healthcare, Novo Nordisk, Sanofi-Aventis, Stago; educational and travel funding from various companies over the years (Amgen SAS, Astra-Zeneca, Bayer Healthcare, Boehringer-Ingelheim, Bristol-Myers Squibb-Pfizer, Daïchi-Sankyo, Fumouze Diagnostics-Sofibel, Glaxo-Smith-Kline, Horiba ABX SAS, Léo Pharma France, LFB-Biomédicaments, Novo Nordisk, Sanofi-Aventis, Stago) and has served as a member on steering boards and scientific advisory boards of Bayer Healthcare, Coagulant Therapeutics, LFB-Biomédicaments, Sanofi-Aventis, Stago.

Author contributions

Jean-Christophe Gris managed part of the included patients, conceived the study, contributed to data analysis and wrote the paper.

Nicolas Molinari and Éric Matzner-Lober contributed to the study design, performed the statistical analyses and contributed to the writing of the paper.

Éva Cochery-Nouvellon, Chloé Bourguignon, Éric Mercier and Sylvie Bouvier performed the laboratory work and generated the laboratory data, and contributed to the writing of the paper.

Isabelle Quéré and Antonia Perez-Martin managed part of the included patients, contributed to data analysis and to the writing of the paper.

ORCID

Jean-Christophe Gris : https://orcid.org/0000-0002-9899-9910

Sylvie Bouvier: https://orcid.org/0000-0001-8808-8841

Nicolas Molinari : https://orcid.org/0000-0002-1786-0088

Éric Matzner-Lober: https://orcid.org/0000-0002-9187-8390

Isabelle Quéré : https://orcid.org/0000-0002-1492-9764

Antonia Pérez-Martin : https://orcid.org/0000-0002-8527-7783

Ethics approval

The study was approved by Nîmes University Hospital' Institutional Review Board and ethics committee (IRB n° 20.09.01) involving data without patient identifiers and performed in accordance with the policy on bioethics and human biological samples of French laws on clinical research and in accordance with the 1996 revised version of the 1975 Helsinki declaration.

REFERENCES

[1] J.M. Connors, Thrombophilia Testing and Venous Thrombosis, N. Engl. J. Med. 377 (23) (2017) 1177-1187.

[2] R.M. Stern, H. Al-Samkari, J.M. Connors, Thrombophilia evaluation in pulmonary embolism, Curr. Opin. Cardiol. 34 (6) (2019) 603-609.

[3] G. Pernod, C. Biron-Andreani, P.E. Morange , et al**.,** Recommendations on testing for thrombophilia in venous thromboembolic disease: a French consensus guideline, J. Mal. Vasc. 34 (3) (2009) 156-203.

[4] C. Kearon, W. Ageno, S.C. Cannegieter, B. Cosmi, G.L. Geersing, P.A. Kyrle; Subcommittees on Control of Anticoagulation, and Predictive and Diagnostic Variables in Thrombotic Disease, Categorization of patients as having provoked or unprovoked venous thromboembolism: guidance from the SSC of ISTH, J. Thromb. Haemost. 14 (7) (2016) 1480-1483.

[5] A. Tripodi, V. Chantarangkul, I. Martinelli, P. Bucciarelli, P.M. Mannucci, A shortened activated partial thromboplastin time is associated with the risk of venous thromboembolism, Blood 104 (12) (2004) 3631-3634.

[6] C. Legnani, S. Mattarozzi, M. Cini, B. Cosmi, E. Favaretto, G. Palareti, Abnormally short activated partial thromboplastin time values are associated with increased risk of recurrence of venous thromboembolism after oral anticoagulation withdrawal, Br. J. Haematol. 134 (2) (2006) 227-232.

[7] T. Koster, F.R. Rosendaal, P.H. Reitsma, P.A. van der Velden, E. Briët, J.P. Vandenbroucke JP, Factor VII and fibrinogen levels as risk factors for venous thrombosis. A case-control study of plasma levels and DNA polymorphisms--the Leiden Thrombophilia Study (LETS), Thromb. Haemost. 71 (6) (1994) 719-722.

[8] K.C. Lounes, C. Soria, S.S. Mirshahi SS, et al, Fibrinogen Alès: a homozygous case of dysfibrinogenemia (gamma-Asp(330)-->Val) characterized by a defective fibrin polymerization site "a", Blood 96 (10) (2000) 3473-3479.

[9] T. Koster, A.D. Blann, E. Briët, J.P. Vandenbroucke, F.R. Rosendaal, Role of clotting factor VIII in effect of von Willebrand factor on occurrence of deep-vein thrombosis, Lancet 345 (8943) (1995) 152-155.

[10] J. O'Donnell, E.G. Tuddenham, R. Manning, G. Kemball-Cook, D. Johnson, M. Laffan, High prevalence of elevated factor VIII levels in patients referred for thrombophilia screening: role of increased synthesis and relationship to the acute phase reaction, Thromb. Haemost. 77 (5) (1997) 825-828.

[11] R.A. Kraaijenhagen, P.S. in't Anker, M.M. Koopman, et al, High plasma concentration of factor VIIIc is a major risk factor for venous thromboembolism, Thromb Haemost 83 (1) (2000) 5-9.

[12] I.M. Rietveld, W.M. Lijfering, S. le Cessie, et al, High levels of coagulation factors and venous thrombosis risk: strongest association for factor VIII and von Willebrand factor, J. Thromb. Haemost. 17 (1) (2019) 99-109.

[13] J.C. Meijers, W.L. Tekelenburg, B.N. Bouma, R.M. Bertina, F.R. Rosendaal, High levels of coagulation factor XI as a risk factor for venous thrombosis, N. Engl. J. Med. 342 (10) (2000) 696-701.

[14] A. van Hylckama Vlieg, I.K. van der Linden, R.M. Bertina, F.R. Rosendaal. High levels of factor IX increase the risk of venous thrombosis, Blood 95 (12) (2000) 3678-3682.

[15] P.A. Kyrle, E. Minar, M. Hirschl, et al, High plasma levels of factor VIII and the risk of recurrent venous thromboembolism, N. Engl. J. Med. 343 (7) (2000) 457-462.

[16] C. Legnani, C. Benilde, C. Michela, F. Mirella, G. Giuliana, P. Gualtiero, High plasma levels of factor VIII and risk of recurrent venous thromboembolism, Br. J. Haematol. 124 (4) (2004) 504-510.

[17] J.F. Timp, W.M. Lijfering, L.E. Flinterman, et al, Predictive value of factor VIII levels for recurrent venous thrombosis: results from the MEGA follow-up study, J. Thromb. Haemost. 13 (10) (2015) 1823-1832.

[18] P.A. Kyrle, L. Eischer, H. Šinkovec, S. Eichinger, Factor XI and recurrent venous thrombosis: an observational cohort study, J. Thromb. Haemost. 17 (5) (2019) 782-786.

[19] A. Weltermann, S. Eichinger, C. Bialonczyk, et al, The risk of recurrent venous thromboembolism among patients with high factor IX levels, J. Thromb. Haemost. 1 (1) (2003) 28-32.

[20] S.R. Poort, F.R. Rosendaal, P.H. Reitsma, R.M. Bertina, A common genetic variation in the 3'-untranslated region of the prothrombin gene is associated with elevated plasma prothrombin levels and an increase in venous thrombosis, Blood 88 (10) (1996) 3698-3703.

[21] M.N. Di Minno, F. Dentali, R. Lupoli, W. Ageno W, Mild antithrombin deficiency and risk of recurrent venous thromboembolism: a prospective cohort study, Circulation 129 (4) (2014) 497-503.

[22] J. Sokol, J.F. Timp, S. le Cessie, et al, Mild antithrombin deficiency and risk of recurrent venous thromboembolism: results from the MEGA follow-up study, J. Thromb. Haemost. 16 (4) (2018) 680-688.

[23] T. Koster, F.R. Rosendaal, E. Briët, et al, Protein C deficiency in a controlled series of unselected outpatients: an infrequent but clear risk factor for venous thrombosis (Leiden Thrombophilia Study), Blood 85 (10) (1995) 2756-2761.

[24] M.C. Pintao, D.D. Ribeiro, I.D. Bezemer, et al., Protein S levels and the risk of venous thrombosis: results from the MEGA case-control study, Blood 122 (18) (2013) 3210-3219.

[25] G. Lavigne, E. Mercier, I. Queré, M. Dauzat, J.C. Gris, Thrombophilic families with inheritably associated high levels of coagulation factors VIII, IX and XI, J. Thromb. Haemost. 1 (10) (2003) 2134-2139.

[26] P. Simioni, S. Cagnin, F. Sartorello, et al, Partial F8 gene duplication (Factor VIII Padua) associated with high factor VIII levels and familial thrombophilia, Blood 137(17) (2021)2383-2393.

[27] I.M. Rietveld, W.M. Lijfering, S. le Cessie, et al, High levels of coagulation factors and venous thrombosis risk: strongest association for factor VIII and von Willebrand factor, J. Thromb. Haemost. 17 (1) (2019) 99-109.

[28] H. Wang, F.R. Rosendaal, M. Cushman, A. van Hylckama Vlieg, Procoagulant factor levels and risk of venous thrombosis in the elderly, J. Thromb. Haemost. 19 (1) (2021) 186-193.

[29] J.F. Timp, S.K. Braekkan, W.M. Lijfering, et al, Prediction of recurrent venous thrombosis in all patients with a first venous thrombotic event: The Leiden Thrombosis Recurrence Risk Prediction model (L-TRRiP), PLoS Med 16 (10) (2019) e1002883.