Von Willebrand disease and von Willebrand factor: an old story, a new perspective

Biguzzi, E.F.

Chapter 2

Rise of levels of von Willebrand factor and factor VIII with age: role of genetic and acquired risk factors

Eugenia Biguzzi, Filippo Castelli, Willem M. Lijfering, Suzanne C. Cannegieter, Jeroen Eikenboom, Frits R. Rosendaal, Astrid van Hylckama Vlieg

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Highlights

- Von Willebrand factor (VWF) and factor VIII (FVIII) increase with age
- VWF and FVIII levels are regulated by genetic and acquired factors
- VWF and FVIII increase with age is partially mediated by acquired factors
- Blood group non-O shows a higher increase of VWF mediated by acquired factors

ABSTRACT

Background. Von Willebrand factor (VWF) levels are regulated by genetic and acquired factors. The acquired factors are mostly related to age and could be mediators of the age effect on VWF levels.
Objectives. To disentangle the role of genetic (sex, blood group) and acquired factors (comorbidities, body mass index, reduced kidney function, hormone use, and inflammation) in regulating von Willebrand factor antigen (VWF:Ag) and factor VIII activity (FVIII:C) levels in the normal population.
Methods. Analysis were performed in a large population sample (2923 individuals) from the Multiple Environmental and Genetic Assessment of risk factors for venous thrombosis (MEGA study), after exclusion of individuals with active cancer and women who were pregnant or within nine months postpartum. The increase of VWF:Ag and FVIII:C with age was evaluated by linear regression after the age of 40 years. Analyses were adjusted for acquired factors and stratified for sex and blood group.
Results. VWF:Ag and FVIII:C increased with age: increase per decade of age for VWF:Ag 18 IU/dL (95%CI 15-20) and for FVIII:C 12 IU/dL (95%CI 10-14). After adjustment for acquired factors, the increase per decade was 13 IU/dL (95%CI 10-16) for VWF:Ag and 9 IU/dL (95%CI 6-11) for FVIII:C. The stratified analysis for blood group showed higher increase in the non-O group, but these differences were annulled after adjustment for acquired factors.
Conclusions. VWF:Ag and FVIII:C increase with age. Carriers of blood group non-O present a steeper increase of VWF:Ag and FVIII:C with age, that is mediated by acquired factors.

INTRODUCTION

Von Willebrand factor (VWF) is a multimeric glycoprotein, synthesized by endothelial cells and megakaryocytes. VWF synthesis as monomers is followed by glycosylation, dimerization and multimerization and VWF comprises molecules that contain a variable number of subunits (range 2 to 100), with different molecular weights [1]. VWF produced by the endothelial cells is partly constitutively secreted into the plasma, but VWF is mainly stored in Weibel-Palade bodies and released after endothelial stimulation. In platelets VWF is stored in the α-granules and secreted upon platelet activation. VWF promotes platelet adhesion and aggregation in primary hemostasis, it regulates FVIII levels in blood, by circulating in complex with it and protecting it from inactivation [1]. It is also involved in inflammation, angiogenesis and wound healing [2, 3]. VWF plasma levels (and consequently FVIII concentration) are the result of the equilibrium between VWF synthesis and its clearance. VWF transcription is regulated by several factors that can enhance (GATA, Ets, H1, NFAT5) or downregulate it (micro-RNA 24) [4]. Hypoxic conditions upregulate VWF transcription in endothelial cells and megakaryocytes, while there is no direct role for inflammatory cytokines and the JAK-STAT signaling pathway in VWF transcription [4]. VWF clearance occurs mainly in the liver and spleen by macrophages [1], which could be evaluated in vivo by analyzing VWF half-life after desmopressin administration, that determines release of VWF from storage. The main determinants of the large variation in VWF half-life were glycosylation differences across blood groups [5].

VWF plasma levels are regulated by genetic factors (among which ABO blood group accounts for around 20% of the genetic variability) [6] and acquired factors such as exercise, hormones, diabetes mellitus, inflammation, thyroid, liver and renal function. [7-11] The acquired factors are mostly related to age and could therefore be mediators of the well-established effect of age on VWF and FVIII levels in the normal population. The aim of our study was to disentangle the role of genetic (sex, blood group) and acquired factors (comorbidities, body mass index [BMI], reduced kidney function, hormone use, and inflammation) in regulating VWF and FVIII levels in a large population sample from the Multiple Environmental and Genetic Assessment of risk factors for venous thrombosis (MEGA study).

METHODS

Study design and data collection.

The MEGA study (Multiple Environmental and Genetic Assessment of risk factors for venous thrombosis study) is a case-control study that enrolled 4956 consecutive patients aged 18-70 years with a first event of venous thromboembolism from 6 anticoagulant clinics in the Netherlands between March 1999 and September 2004. [12] The MEGA study enrolled as controls 3297 partners of patients and 3000 individuals recruited via random-digit-dialing (RDD) method, without venous thromboembolism, matched on sex and age with the patients and from the same geographical area.

At enrolment all participants filled in a detailed questionnaire on medical history and current use of drugs. Information on comorbidities (liver disease, kidney disease, rheumatoid arthritis, multiple sclerosis, heart failure, haemorrhagic stroke, diabetes mellitus, hypothyroidism, hyperthyroidism, chronic bronchitis, pulmonary emphysema) were collected from these questionnaires as well as data regarding arterial thrombosis (angina pectoris, myocardial infarction, ischemic infarction and stroke, transient ischemic attack and peripheral arterial disease). BMI was calculated from self-reported body weight and height (kilograms/meters^2). Since arterial hypertension was not among the reported illnesses, presence of high blood pressure was derived from the use of calcium-channel blockers, beta-blockers (carvedilol, nebivolol, labetalol) and alpha-blockers (doxazosin). Furthermore, some drugs were considered a proxy for hypertension in case of absence of an alternative indication: angiotensin-converting enzyme inhibitors in the absence of heart failure, sartans (angiotensin II receptor blockers) in the absence of heart failure and kidney disease, diuretics in the absence of heart failure, kidney disease and liver disease. The use of atenolol, bisoprolol, metoprolol, propranolol, sotalol, nadolol or pindolol was not considered proxy for hypertension, since these drugs can be prescribed for tachycardia and esophageal varices. Hormone use was defined as either oral contraceptive or hormonal replacement therapy use.

For the present study, we included the control group (partners of patients or RDD controls) of the MEGA study representing a sample of the general population. In order to avoid transient conditions associated with increased levels of VWF and FVIII, controls were excluded if they had an active cancer in the previous 5 years and if they were pregnant (or within 9 months after delivery). For logistic reasons blood samples were taken until June 2002 leaving a total of 2943 controls (1483 partners and 1460

RDD controls). After exclusion of 57 participants who reported cancer (and 5 with missing data), 47 women who were pregnant or within 9 months after delivery (and 8 with missing data) and 3 individuals with no available data on VWF:Ag or FVIII:C, we included 2823 participants in the current analysis.
This study was approved by the Ethics Committee of the Leiden University Medical Center, and written consent was obtained from all participants.

Blood collection, laboratory measurements and data collection.

Factor VIII activity (FVIII:C) was measured by a mechanical clot detection method and von Willebrand factor antigen (VWF:Ag) with an immunoturbidimetric method, the STA Liatest kit. Both tests were performed on a STA-R coagulometer, according to the manufacturer instructions (Diagnostica Stago, France). Samples were pre-diluted (1:40 for FVIII assay and 1:8 for the VWF assay) to be within the linear part of the curve, even with high levels (no patients affected by hemophilia A or von Willebrand disease were enrolled in the study). The assays were linear up to 600IU/dL for FVIII:C and 420 IU/dL for VWF:Ag.
The mean intra-assay and inter-assay coefficient of variations were 3.6% and 2.6% for VWF:Ag. 3.7% and 8.9% for FVIII:C. Serum creatinine was measured enzymatically (Roche diagnostics, Germany) and estimated glomerular filtration rate (eGFR) was estimated using the Modification of Diet in Renal Disease study equation (MDRD). [13] C reactive protein (CRP) was measured by automated particle-enhanced immunoturbidimetric assay (Tina-quant® CRP, Roche Diagnostics, Germany). Blood group was genetically determined using the Taqman system (Applied Biosystems, USA) and the following polymorphisms: 20146G/- (rs176719), 21463C/G (rs7853989), 21867A/G (rs8176749) and 21996C/- (rs8176750).

Statistical methods.

Categorical data are presented as frequencies and percentages. Continuous data are presented as mean and standard deviation (SD). FVIII:C and VWF:Ag distribution were visually inspected and considered normal.
Since CRP levels were skewed, log transformation was applied to this variable, obtaining a normal distribution. Multiple linear regression analysis was used to evaluate the association between age and levels of FVIII:C and VWF:Ag. Participants were stratified in age groups and mean differences (and 95% confidence intervals, CI) of FVIII:C and VWF:Ag were calculated, using the youngest group as reference. The increase of FVIII:C and VWF:Ag with age was also analysed with age as a continuous

variable after the age of 40 years (linear part of the curve), where we report the β coefficient and its 95% CI. The analyses were first adjusted for individual mediators (comorbidities, arterial thrombosis, estrogen use, BMI, renal function or CRP levels) and subsequently adjusted for all mediators combined. BMI, renal function and CRP were added as continuous variables. A stratified analysis for sex and blood group (O versus non-O) was performed to analyse genetic confounders.
The statistical analysis was performed using SPSS version 26 (IBM, Armonk, NY, USA).

RESULTS

Table 1 shows the clinical characteristics of participants: in total 2823 individuals were evaluated and they represent a sample of the general population, which was restricted to individuals between 18 and 70 years. The mean age was 49 years (SD 12) and the mean levels were for VWF:Ag 112 IU/dL (SD 47) and for FVIII:C 112 IU/dL (SD 38).
Our study included some individuals with low levels of VWF, who did not have a history of bleeding symptoms (59 people with VWF:Ag ≤50 IU/dL, 2% of the whole population). The vast majority (n=51, 86%) were blood group O.
Only a minority of the study population (13%) was obese (defined as BMI >30 kg/m2) or were affected by comorbidities (16%) or arterial thrombosis (5%), while 26% of the women reported hormone use.
Table 2 shows the mean levels of VWF:Ag and FVIII:C in strata of increasing age, as well as the distribution of the potential mediators (comorbidities, arterial thrombosis, renal function, CRP and hormone use) across these strata. VWF:Ag and FVIII:C increased with age, as well as the prevalence of comorbidities, arterial thrombosis, reduced renal function, and increased levels of CRP. As expected hormone use was most frequent in the youngest group.
Mean differences (and 95% CI) of VWF:Ag and FVIII:C in the whole population across age groups are shown in Table 3, before and after adjustment for comorbidities, BMI, renal function, CRP levels and hormone use. For both VWF and FVIII, the increase with age was most pronounced after the age of 50 years and was almost negligible before 50 years. The adjusted increase of VWF:Ag, when compared with the reference age group (18-30 years), was 10 IU/dL (95% CI 3 to 17) for age group 50-60 years and 27 IU/dL (95% CI 19 to 35) for age group 60-70 years. For FVIII:C these increases were 7 IU/dL (95% CI 1 to 12) and 18 IU/dL (95% CI 12 to 24).

Table 1. Clinical Characteristics

	Controls
Total	(n=2823)
Population	
Male, nº (%)	1364 (48)
Age at enrolment (years), mean (SD)	49 (12)
Clinical risk factors	
Hormone use (in women), no. (%)	358 (26)
BMI, mean (SD)	25.61 (4)
Clinical chemistry	
C reactive protein (mg L^{-1}), mean (SD)	2.83 (5.68)
eGRF (ml min^{-1}), mean (SD)	87 (17)
Genetics	
Blood Group non-O, no (%)	1502 (53)
Coagulation factors	
von Willebrand factor antigen (IU dL^{-1}), mean (SD)	112 (47)
FVIII activity (IU dL^{-1}), mean (SD)	112 (38)
Comorbidities, nº (%)*	444 (16)
Arterial thrombosis	131 (5)

eGFR: estimated glomerular filtration rate;BMI: body mass index
Some variables have missing data (12 for comorbidities, 77 for BMI, 8 for eGFR, 6 for CRP, 78 for hormone use)
* Some individuals have >1 comobidity: liver disease (n=11), kidney disease (n=14), rheumatoid arthritis (n=61), multiple sclerosis (n=9), heart failure (n=30), haemorrhagic stroke (n=6), diabetes mellitus (n=87), hypothyroidism or hyperthyroidism (n=82), chronic bronchitis (n=81), pulmonary emphysema (n=19), angina pectoris (n=28), myocardial infarction (n=60), ischemic infarction or stroke (n=20), transient ischemic attack (n=26) and peripheral arterial disease (n=26). In addition, high blood pressure on therapy (n=275), high blood pressure or atenolol use (n=336)

Table 2. Mean levels (and SD) of VWF:Ag and FVIII:C and distribution of comorbidities, arterial thrombosis, renal function, CRP and hormone use across age groups.

Age (years)	Number of participants, n (%)	VWF:Ag IU dL^{-1} Mean (SD)	FVIII:C IU dL^{-1} Mean (SD)	Comorbidities n (%)	Arterial Thrombosis n (%)	BMI >30 kg square m^{-1} n (%)	eGRF <60 ml min^{-1} n (%)	CRP >5 mg L^{-1} n (%)	Hormone use N (% of women)
18-30	223 (8)	97 (35)	101 (35)	12 (6)	0	16 (8)	1 (0.4)	25 (11)	68 (62)
30-40	480 (17)	97 (34)	101 (32)	27 (6)	0	52 (11)	2 (0.4)	60 (13)	88 (40)
40-50	677 (24)	101 (36)	105 (35)	77 (11)	11 (2)	65 (10)	6 (0.9)	92 (14)	107 (31)
50-60	847 (30)	115 (43)	114 (36)	159 (19)	44 (5)	118 (14)	20 (2.4)	115 (14)	68 (15)
60-70	596 (21)	136 (62)	129 (43)	169 (28)	76 (13)	100 (18)	48 (8.1)	98 (17)	27 (10)

Table 3. Mean differences (and 95% CI) of FVIII:C and VWF:Ag (unadjusted and adjusted for comorbidities, BMI, renal function, CRP and hormone use. The adjustment for BMI, eGFR and log transformed CRP was performed as continuous variables.

	Age (years)	**Number of controls, n**	**Mean levels (IU dL^{-1})**	**Mean difference IU dL^{-1} (95% CI)**						
				Unadjusted	**Adjusted for comorbidities**	**Adjusted for BMI**	**Adjusted for eGFR**	**Adjusted for CRP**	**Adjusted for hormone use**	**Adjusted for comorbidities, BMI, eGFR, CRP, hormone use**
VWF:Ag	18-30	223	97 (35)	Reference	Reference	Reference	Reference	Reference	Reference	Reference
	30-40	480	97 (34)	0 (-6 to 8)	1 (-6 to 8)	1 (-7 to 8)	-1 (--8 to 6)	0 (-7 to 7)	1 (-6 to 8)	-1 (-8 to 6)
	40-50	677	101 (36)	5 (-2 to 12)	4 (-2 to 11)	4 (-3 to 11)	2 (-5 to 9)	4 (-3 to 10)	6 (-1 to 12)	0 (-7 to 7)
	50-60	847	115 (43)	18 (11 to 23)	17 (10 to 23)	17 (10 to 23)	13 (6 to 20)	16 (9 to 22)	19 (13 to 26)	10 (3 to 17)
	60-70	596	136 (62)	39 (32 to 46)	37 (30 to 44)	38 (31 to 45)	33 (26 to 40)	36 (29 to 43)	40 (33 to 47)	27 (19 to 35)
FVIII:C	18-30	223	101 (35)	Reference	Reference	Reference	Reference	Reference	Reference	Reference
	30-40	480	101 (32)	0 (-6 to 5)	0 (-6 to 5)	0 (-7 to 5)	-2 (-8 to 4)	-1 (-6 to 5)	1 (-5 to 7)	-2 (-8 to 4)
	40-50	677	105 (35)	4 (-2 to 9)	3 (-2 to 9)	3 (-2 to 9)	1 (-5 to 6)	3 (-2 to 9)	5 (0 to 11)	0 (-5 to 6)
	50-60	847	114 (36)	13 (8 to 18)	12 (7 to 18)	12 (6 to 17)	8 (3 to 14)	11 (6 to 17)	15 (9 to 20)	7 (1 to 12)
	60-70	596	129 (43)	27 (22 to 33)	26 (20 to 32)	27 (21 to 33)	21 (15 to 27)	25 (20 to 31)	29 (23 to 35)	18 (12 to 24)

When we analysed VWF:Ag and FVIII:C continuously for the age 40-70 years (linear part of the curve), the increase per decade of age (β) for VWF:Ag was 18 IU/dL (95%CI 15-20) and for FVIII:C was 12 IU/dL (95%CI 10-14) (Table 4). In the full model the increase of VWF:Ag and FVIII:C per decade of age (β) was 13 IU/dL (95%CI 10-16) and 9 IU/dL (95%CI 6-11).

A detailed adjusted analysis was also performed with specific comorbidities, i.e, arterial thrombosis only; comorbidities after exclusion of arterial thrombosis; comorbidities and high blood pressure; comorbidities, high blood pressure and atenolol use. All these analyses were in line with the main results (data not shown).

A stratified analysis was performed for blood group (O versus non-O).

Figure 1 shows the increase of VWF:Ag and FVIII:C with age, which was attenuated after adjustment for comorbidities, BMI, hormone use, eGFR and CRP (full model). As expected, individuals with blood group non-O had higher levels of VWF:Ag and FVIII:C than those with blood group O.

Table 5 shows the results of multiple linear regression analysis stratified for blood group: the increases of VWF:Ag and FVIII:C per decade of age (after 40 years) were higher in blood group non-O (β: 20 IU/dL, 95% CI 16-23, for VWF:Ag and 13 IU/dL, 95% CI 10-16, for FVIII:C), than in blood group O (β: 14 IU/dL, 95% CI 16-16, for VWF:Ag and 11 IU/dL, 95% CI 9-13, for FVIII:C), but this differences were annulled by the adjustment in the full model. Table 6 shows the mean differences of VWF:Ag and FVIII:C across age groups (with the youngest group as reference), confirming in both blood group O and non-O a more evident increase of VWF:Ag and FVIII:C after the age of 50 years.

Stratification was also performed for sex and blood group simultaneously, confirming the overall analysis (supplementary tables 1-4).

The linear regression analysis of continuous VWF and FVIII was also performed in the whole group of normal individuals, confirming the increase with age, albeit with lower β estimates, as expected, since no increase was shown in the younger age groups (data not shown).

DISCUSSION

In the present study we analysed the effect of age on VWF:Ag and FVIII:C levels in a large sample from the general Dutch population. We found an increase of both factors with age (β coefficient 18 IU/dL per decade for VWF:Ag and 12 IU/dL per decade for FVIII:C, after the age of 40 years).

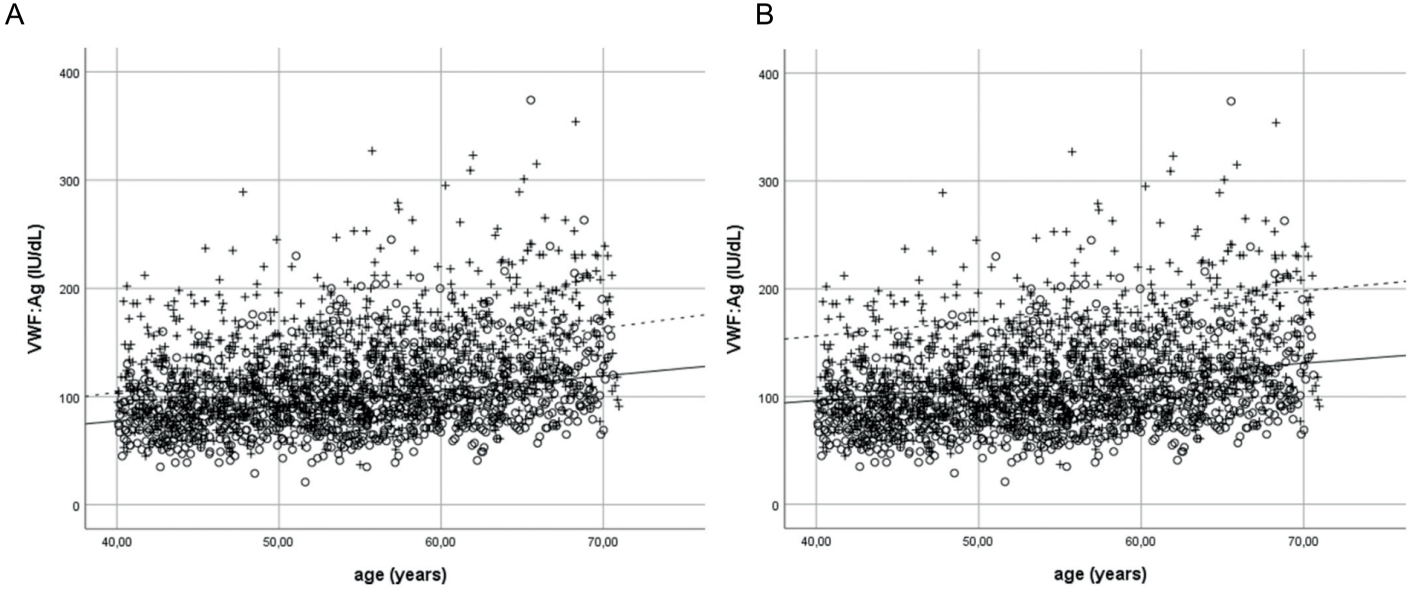
Table 4. Increase of FVIII:C and VWF:Ag as continuous variables with age (beta coefficients for decade are shown), calculated in individuals age 40-70 years.

	Beta coefficient (95% CI) for decade (IU dL^{-1})						
	Unadjusted	**Adjusted for comorbidities**	**Adjusted for BMI**	**Adjusted for eGFR**	**Adjusted for CRP**	**Adjusted for hormone use**	**Adjusted for comorbidities, BMI, blood group, eGFR, CRP, hormone use**
VWF:Ag	18 (15 to 20)	17 (14 to 19)	17 (15 to 20)	16 (13 to 18)	16 (13 to 19)	17 (15 to 20)	13 (10 to 16)
FVIII:C	12 (10 to 14)	12 (10 to 14)	12 (10 to 14)	11 (9 to 13)	12 (10 to 14)	12 (10 to 14)	9 (6 to 11)

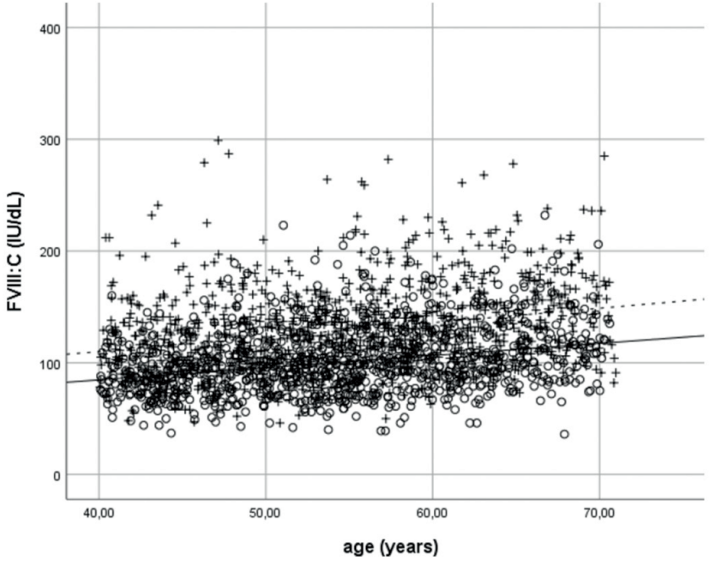
Table 5. Increase of VWF:Ag and FVIII:C as continuous variables with age (beta coefficients for decade are shown), stratified by blood group, calculated in individuals age 40-70 years.

		Beta coefficient (95% CI) for decade (IU dL^{-1})						
		Unadjusted	**Adjusted for comorbidities**	**Adjusted for BMI**	**Adjusted for eGFR**	**Adjusted for CRP**	**Adjusted for hormone use**	**Adjusted for comorbidities, BMI, blood group eGFR, CRP, hormone use**
VWF:Ag	*Blood group O*	14 (11 to 16)	13 (11 to 16)	13 (11 to 16)	13 (11 to 16)	13 (10 to 15)	14 (11 to 16)	12 (9 to 14)
	Blood group non-O	20 (16 to 23)	19 (15 to 23)	19 (16 to 23)	17 (13 to 21)	18 (15 to 22)	20 (16 to 24)	14 (10 to 18)
FVIII:C	*Blood group O*	11 (9 to 13)	11 (8 to 13)	10 (8 to 13)	10 (7 to 12)	10 (8 to 12)	11 (8 to 13)	8 (6 to 10)
	Blood group non-O	13 (10 to 16)	13 (10 to 16)	13 (10 to 16)	11 (10 to 16)	12 (9 to 15)	13 (10 to 16)	9 (6 to 12)

Figure 1: Levels of VWF:Ag (A and B) and FVIII (C and D) distributed by age, stratified by blood group (blood group O indicated by open circles and continuous line, blood group non-O indicated by crosses and dotted line). Unadjusted regression lines are presented in A and C; adjusted regression line (for comorbidies, BMI, eGFR, CRP and hormone use) are presented in B and D.
Four samples were excluded from the graph for VWF:Ag (>400 IU/dL) and one for FVIII:C (>400 IU/dL).



C


FVIII:C (IU/dL)
400
300
200
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0
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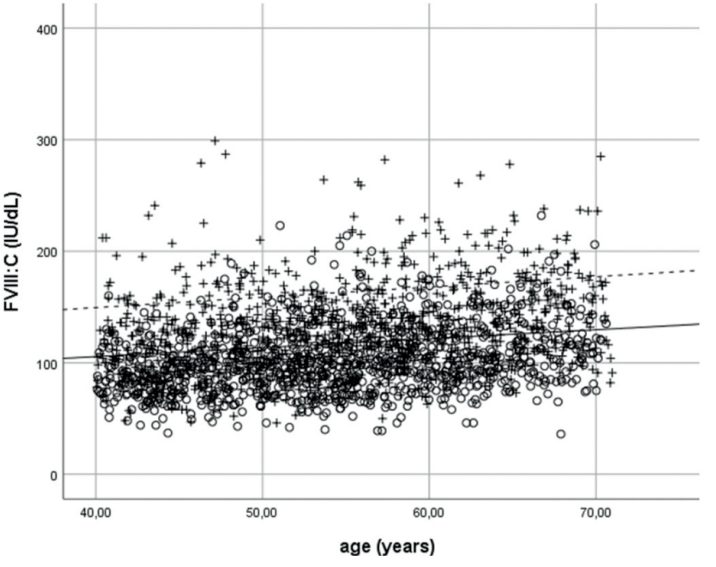
70,00
age (years)

D


FVIII:C (IU/dL)
400
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60,00
70,00
age (years)

Table 6. Mean differences (and 95% CI) of VWF:Ag and FVIII:C stratified by blood group (unadjusted and adjusted for comorbidities, BMI, renal function, CRP and hormone use). The adjustment for BMI, eGFR and log transformed CRP was performed as continuous variables.

	Blood group	Age (years)	Number of controls n	Mean (SD) IU dL^{-1}	Mean difference (95% CI) IU dL^{-1}						
					Unadjusted	Adjusted for comorbidities	Adjusted for BMI	Adjusted for eGFR	Adjusted for CRP	Adjusted for hormone use	Adjusted for comorbidities, BMI, eGFR, CRP, hormone use
VWF:Ag	*Blood*	18-30	101	79 (19)	Reference	Reference	Reference	Reference	Reference	Reference	Reference
	group	30-40	214	81 (27)	2 (-5 to 9)	2 (-5 to 10)	2 (-5 to 9)	1 (-6 to 7)	2 (-5 to 10)	3 (-4 to 10)	2 (-5 to 10)
	O	40-50	322	85 (25)	6 (-1 to 13)	5 (-2 to 12)	5 (-2 to 12)	4 (-3 to 11)	5 (-1 to 12)	7 (0 to 14)	5 (-3 to 12)
		50-60	412	98 (34)	19 (12 to 25)	18 (11 to 25)	18 (11 to 24)	17 (10 to 24)	17 (11 to 24)	20 (13 to 27)	16 (9 to 23)
		60-70	266	112 (39)	32 (25 to 39)	30 (24 to 38)	31 (23 to 38)	30 (22 to 37)	30 (23 to 37)	33 (26 to 41)	27 (20 to 34)
	Blood	18-30	121	111 (38)	Reference	Reference	Reference	Reference	Reference	Reference	Reference
	group	30-40	266	110 (33)	-1 (-11 to 9)	-1 (-11 to 10)	-1 (-12 to 9)	-3 (-14 to 7)	-2 (-12 to 8)	0 (-10 to 11)	-4 (-15 to 6)
	non-O	40-50	352	116 (38)	6 (-4 to 15)	6 (-5 to 15)	4 (-6 to 14)	1 (-9 to 11)	4 (-6 to 14)	6 (-4 to 16)	-2 (-12 to 8)
		50-60	434	130 (45)	20 (10 to 29)	18 (8 to 28)	18 (8 to 28)	12 (2 to 22)	16 (7 to 26)	21 (11 to 31)	7 (-3 to 18)
		60-70	329	155 (69)	45 (35 to 55)	42 (32 to 53)	43 (32 to 53)	35 (24 to 45)	40 (31 to 50)	46 (36 to 57)	26 (16 to 38)
FVIII:C	*Blood*	18-30	101	87 (26)	Reference	Reference	Reference	Reference	Reference	Reference	Reference
	group	30-40	214	88 (27)	1 (-6 to 8)	1 (-6 to 8)	1 (-6 to 7)	-1 (-7 to 6)	1 (-5 to 8)	2 (-4 to 9)	1 (-6 to 8)
	O	40-50	322	90 (24)	3 (-3 to 10)	3 (-3 to 9)	3 (-3 to 9)	1 (-5 to 7)	3 (-3 to 9)	5 (-1 to 12)	2 (-4 to 10)
		50-60	412	101 (31)	15 (8 to 21)	14 (7 to 20)	13 (7 to 19)	11 (5 to 17)	13 (7 to 19)	17 (11 to 24)	11 (5 to 18)
		60-70	266	111 (31)	24 (17 to 30)	23 (16 to 29)	23 (16 to 29)	19 (12 to 26)	22 (15 to 28)	26 (19 to 33)	18 (11 to 25)
	Blood	18-30	121	113 (36)	Reference	Reference	Reference	Reference	Reference	Reference	Reference
	group	30-40	266	112 (32)	-2 (-10 to 7)	-2 (-10 to 7)	-2 (-10 to 6)	-4 (-12 to 4)	-2 (-10 to 6)	0 (-8 to 8)	-4 (-12 to 5)
	non-O	40-50	352	119 (37)	6 (-2 to 13)	5 (-3 to 13)	5 (-3 to 13)	2 (-6 to 10)	5 (-3 to 12)	7 (-1 to 15)	1 (-7 to 9)
		50-60	434	127 (36)	14 (6 to 21)	13 (5 to 21)	13 (5 to 21)	8 (0 to 15)	12 (4 to 19)	15 (7 to 23)	5 (-3 to 13)
		60-70	329	144 (45)	30 (23 to 38)	29 (21 to 37)	30 (22 to 38)	22 (14 to 21)	28 (20 to 36)	32 (23 to 40)	19 (10 to 28)

Adjustment for BMI, comorbidities, and hormone use did not affect the association between FVIII:C and VWF:Ag and age, while both the adjustment for eGFR as well as the adjustment for CRP, individually, led to a reduction in the strength of the association between FVIII:C and VWF:Ag and age (of approximately 25%). The analysis by age groups in our study showed that the increase is almost negligible until 50 years and becomes evident after this age. The increase of FVIII:C with age as shown in the current study was already described in 1976 in a large group of blood donors [14] and these data were confirmed by subsequent studies for both FVIII:C and for VWF:Ag. [15, 16] We could not compare our finding with the ARIC study, since this study enrolled only individuals over the age of 45 years.[15] The third Glasgow MONICA survey did not report the same trend after the age of 50 years. This could be due to differences in the prevalence of the confounders (not described in the MONICA study) or to different characteristics of the Glasgow population, that comes from an area of relatively high cardiovascular risk. [16]

VWF levels were evaluated in twins[17] and genetics were found to explain 66% of their plasma variations: one third of VWF genetic variation was due to ABO blood group. Several studies, which evaluated the possible mechanism explaining higher levels of VWF in individuals with blood group non-O blood, described an increased clearance of VWF in individuals with blood group O. [5, 6, 18] Increased clearance of VWF in blood group O could be due to specific characteristics of VWF or due to the clearance system of individuals who are of blood group O. This was evaluated by Groeneveld et al, in a mouse model and in patients affected by type 3 and type 1 von Willebrand disease infused with VWF/FVIII concentrate, finding a shorter half-life of VWF in blood group O individuals. [19] On the other hand, ABO blood group could play a role through the terminal sugars of VWF, making it more susceptible to proteolysis by ADAMTS-13. [20, 21]

A recent report [22] confirmed the increased clearance of VWF in blood group O, but found also increased secretion of VWF in blood group non-O, evaluated by the levels of VWF propeptide and confirmed by angiopoietin-2 levels in a subgroup of patients. The same authors also found that the difference in levels of VWF and FVIII across blood-groups increases with age. In our study after stratification for blood-group, the increase with age of VWF:Ag and FVIII:C was confirmed to be higher in non-O blood group than in blood group O, but this difference was lost after adjustment for the acquired variables (comorbidities, BMI, hormone use, eGFR and CRP levels).

Interestingly, a role of comorbidities in increasing levels of VWF with age was previously observed in a large group of patients affected by type 1 von Willebrand

disease, [23] where the adjustment for comorbidities annulled the observed effect of increasing age on VWF levels. In contrast, in our analysis, comorbidities did not modify the observed effect of age on levels of VWF and FVIII. Since high blood pressure was not among the self-reported comorbidities, we also performed an analysis using anti-hypertensive drug use as proxy for high blood pressure, but even in this case the adjustment for comorbidities (including high blood pressure) did not affect the estimates of the age-related increase of VWF and FVIII. It is possible that our analysis underestimated the prevalence of high blood pressure for 2 reasons: 1) the use of some drugs (such as atenolol and others) was not considered since they can be used also for other indications, 2) probably some individuals with high blood pressure were not on any therapy.

Reduced glomerular filtration, evaluated by the eGFR_mdrd formula, together with elevated CRP, was found the most important mediator of age in the increase of VWF and FVIII in our analysis. Since VWF and FVIII are elevated in inflammation, the role of CRP was expected. [4] Elevated VWF and FVIII in reduced renal function are not likely due to decreased clearance by the kidney itself, because VWF and FVIII are cleared by the reticuloendothelial system of the liver and spleen. High levels of VWF and FVIII could be a manifestation of inflammation and vascular injury associated to decreased renal function, that is often the endpoint of diseases characterized by endothelial damage (such as diabetes mellitus, cardiovascular disease and atherosclerosis). Unfortunately, we could not analyse specifically the role of liver function in the clearance of VWF with age, because only few individuals reported liver disease and no measurements of transaminases and albumin were available.

Our study included some individuals with low levels of VWF, who did not have a history of bleeding symptoms. We believe this does not bias our results, since they are part of the normal distribution of the population. Previous studies showed an increase of VWF even in patients with low levels of VWF and bleeding symptoms or in patients affected by type 1 von Willebrand disease. [24, 25]

A major strength of our study was the large group of normal individuals that allowed us to stratify for sex and blood group and to analyse the role of hormone use, comorbidities, BMI, kidney function and CRP in the age-related increase of VWF and FVIII. Our study's limitations are the use of self-reported information on a limited number of comorbidities, the use of self-reported anti-hypertensive drug use as a proxy of high blood pressure, and the upper age limit of 70 years. It would be of interest, especially in consideration of the current longer life-expectancy, to evaluate also older age groups. Moreover, the present study could not add any data on the pathogenic

mechanism of increased levels of VWF and FVIII with age, since no data on VWF propeptide, ADAMTS-13 or liver function were available in our study. Finally, the role of menopause in women could not be evaluated, but adjustment for hormone use (oral contraceptive or hormonal replacement) was performed. Menopausal status may be relevant since the increase of levels with age is more evident after 50 years.

CONCLUSIONS

Our study confirms the increase of VWF:Ag and FVIII:C with age. Blood group non-O presents a higher increase of VWF:Ag and FVIII with age, which is mediated by acquired risk factors. The increase of both proteins with age is partially mediated by renal function and CRP, that could be related to increased secretion of VWF and FVIII, while ABO blood group could play a major role in the clearance regulation.

Declaration of competing interest

J. Eikenboom received research support from CLS Behring and fees for educational activities from Roche, all funding and fees paid to the institution.

E. Biguzzi received fees for educational activities from Takeda, paid to the institution.

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Supplementary table 1 (first part). Mean differences (and 95% CI) of VWF:Ag, stratified by sex and blood group (unadjusted and adjusted for comorbidities, BMI, renal function, CRP and hormone use). The adjustment for BMI, eGFR and log transformed CRP was performed as continuous variables.

VWF:Ag											
	Blood group	**Age (years)**	**Number of controls n**	**Mean VWF:Ag (IU dL^{-1})**	**Mean difference (95% CI) IU dL^{-1}**						
					Unadjusted	**Adjusted for comorbidities**	**Adjusted for BMI**	**Adjusted for eGFR**	**Adjusted for CRP**	**Adjusted for hormone use**	**Adjusted for comorbidities, BMI, eGFR, CRP, hormone use**
Males	*Blood group O*	18-30	45	76 (18)	Reference	Reference	Reference	Reference	Reference	-	Reference
		30-40	110	80 (25)	4 (-7 to 14)	4 (-7 to 14)	4 (-7 to 14)	2 (-9 to 12)	3 (-7 to 13)	-	1 (-9 to 11)
		40-50	157	83 (25)	7 (-3 to 17)	7 (-3 to 17)	7 (-3 to 17)	5 (-5 to 15)	6 (-4 to 15)	-	4 (-6 to 13)
		50-60	180	99 (34)	22 (13 to 32)	22 (12 to 32)	22 (12 to 32)	19 (9 to 29)	19 (9 to 28)	-	17 (7 to 26)
		60-70	144	110 (35)	34 (24 to 44)	33 (23 to 43)	32 (22 to 42)	29 (19 to 40)	29 (19 to 39)	-	25 (14 to 35)
	Blood group non-O	18-30	63	111 (45)	Reference	Reference	Reference	Reference	Reference	-	Reference
		30-40	139	105 (30)	-5 (-20 to 9)	-5 (-20 to 10)	-5 (-19 to 10)	-9 (-24 to 5)	-9 (-23 to 5)	-	-10 (-24 to 4)
		40-50	151	121 (42)	11 (-4 to 25)	11 (-4 to 26)	11 (-3 to 26)	5 (-10 to 19)	7 (-7 to 21)	-	4 (-11 to 18)
		50-60	209	129 (50)	18 (4 to 32)	18 (4 to 32)	19 (5 to 33)	10 (-4 to 25)	10 (-3 to 24)	-	5 (-9 to 20)
		60-70	162	155 (65)	44 (30 to 58)	43 (29 to 58)	45 (30 to 60)	34 (19 to 49)	34 (20 to 48)	-	25 (10 to 41)

Supplementary table 1 (second part). Mean differences (and 95% CI) of VWF:Ag, stratified by sex and blood group (unadjusted and adjusted for comorbidities, BMI, renal function, CRP and hormone use). The adjustment for BMI, eGFR and log transformed CRP was performed as continuous variables.

VWF:Ag											
	Blood group	**Age (years)**	**Number of controls n**	**Mean VWF:Ag (IU dL^{-1})**	**Mean difference (95% CI) IU dL^{-1}**						
					Unadjusted	**Adjusted for comorbidities**	**Adjusted for BMI**	**Adjusted for eGFR**	**Adjusted for CRP**	**Adjusted for hormone use**	**Adjusted for comorbidities BMI, eGFR, CRP, hormone use**
Females	*Blood group O*	18-30	56	82 (19)	Reference	Reference	Reference	Reference	Reference	Reference	Reference
		30-40	104	83 (30)	1 (-9 to 12)	1 (-9 to 12)	1 (-9 to 12)	1 (-9 to 12)	2 (-8 to 12)	2 (-8 to 13)	3 (-8 to 14)
		40-50	165	87 (25)	5 (-5 to 14)	4 (-6 to 14)	4 (-6 to 14)	4 (-6 to 14)	5 (-6 to 14)	6 (-4 to 17)	5 (-6 to 15)
		50-60	232	98 (33)	16 (6 to 25)	14 (5 to 23)	15 (5 to 24)	15 (5 to 25)	16 (5 to 25)	18 (8 to 28)	15 (4 to 25)
		60-70	122	114 (43)	32 (22 to 42)	29 (19 to 39)	31 (21 to 42)	31 (20 to 42)	32 (20 to 42)	34 (22 to 44)	29 (17 to 40)
	Blood group non-O	18-30	58	111 (30)	Reference	Reference	Reference	Reference	Reference	Reference	Reference
		30-40	127	115 (36)	4 (-11 to 19)	4 (-11 to 18)	3 (-12 to 18)	3 (-11 to 18)	5 (-10 to 19)	7 (-8 to 22)	3 (-12 to 18)
		40-50	201	113 (35)	2 (-12 to 15)	-1 (-15 to 13)	-1 (-15 to 13)	-1 (-15 to 12)	2 (-11 to 16)	3 (-11 to 18)	-6 (-20 to 9)
		50-60	225	132 (40)	21 (7 to 34)	18 (5 to 32)	17 (3 to 31)	14 (0 to 28)	20 (7 to 34)	24 (10 to 39)	10 (-5 to 25)
		60-70	167	156 (74)	45 (31 to 59)	41 (27 to 56)	41 (26 to 55)	36 (21 to 50)	45 (11 to 59)	49 (33 to 64)	28 (12 to 45)

Supplementary table 2. Increase of VWF:Ag as continuous variables with age (beta coefficients for decade are shown), stratified by sex and blood group, calculated in individuals age 40-70 years.

VWF:Ag		**Beta coefficient (95% CI) for decade (IU dL^{-1})**						
		Unadjusted	**Adjusted for comorbidities**	**Adjusted for BMI**	**Adjusted for eGFR**	**Adjusted for CRP**	**Adjusted for hormone use**	**Adjusted for comorbidities, BMI, blood group eGFR, CRP, hormone use**
Males	*Blood group O*	13 (9 to 16)	13 (9 to 16)	12 (9 to 15)	8 (5 to 12)	11 (8 to 14)	-	10 (6 to 13)
	Blood group non-O	18 (13 to 24)	18 (12 to 23)	18 (12 to 24)	16 (10 to 22)	15 (9 to 20)	-	12 (6 to 18)
Females	*Blood group O*	15 (11 to 19)	14 (12 to 18)	15 (11 to 19)	15 (12 to 19)	15 (11 to 19)	15 (11 to 19)	14 (10 to 18)
	Blood group non-O	21 (16 to 26)	20 (16 to 25)	20 (15 to 26)	17 (12 to 22)	20 (16 to 26)	21 (16 to 27)	15 (10 to 21)

Supplementary table 3. Mean differences (and 95% CI) of FVIII:C, stratified by sex and blood group (unadjusted and adjusted for comorbidities, BMI, renal function, CRP and hormone use). The adjustment for BMI, eGFR and log transformed CRP was performed as continuous variables.

FVIII:C											
	Blood group	**Age (years)**	**Number of controls n**	**Mean FVIII (IU dL^{-1})**	**Mean difference (95% CI) IU dL^{-1}**						
					Unadjusted	**Adjusted for comorbidities**	**Adjusted for BMI**	**Adjusted for eGFR**	**Adjusted for CRP**	**Adjusted for hormone use**	**Adjusted for comorbidities BMI, eGFR, CRP, hormone use**
Males	*Blood group O*	18-30	45	77 (23)	Reference	Reference	Reference	Reference	Reference	-	Reference
		30-40	110	85 (26)	8 (-2 to 18)	8 (-2 to 18)	8 (-2 to 18)	5 (-4 to 15)	7 (-2 to 17)	-	5 (-4 to 15)
		40-50	157	87 (25)	11 (2 to 20)	10 (1 to 20)	11 (1 to 20)	7 (-2 to 18)	9 (0 to 19)	-	6 (-3 to 16)
		50-60	180	99 (32)	23 (13 to 32)	21 (12 to 31)	22 (12 to 31)	16 (8 to 27)	19 (10 to 28)	-	15 (6 to 24)
		60-70	144	107 (30)	31 (21 to 40)	29 (20 to 39)	30 (20 to 40)	24 (14 to 34)	26 (14 to 36)	-	20 (10 to 30)
	Blood group non-O	18-30	63	110 (43)	Reference	Reference	Reference	Reference	Reference	-	Reference
		30-40	139	107 (29)	-3 (-15 to 9)	-3 (-15 to 9)	-2(-15 to 10)	-8 (-20 to 4)	-5 (-17 to 6)	-	-8 (-20 to 4)
		40-50	151	119 (40)	10 (-2 to 21)	10 (-2 to 22)	11 (-2 to 23)	3 (-9 to 15)	7 (-4 to 19)	-	3 (-10 to 15)
		50-60	209	120 (36)	10 (-1 to 22)	11 (-1 to 22)	11 (0 to 23)	2 (-10 to 14)	6 (-5 to 18)	-	-1 (-13 to 11)
		60-70	162	141 (51)	31 (20 to 43)	31 (19 to 43)	33 (21 to 45)	20 (8 to 32)	26 (14 to 23)	-	16 (3 to 29)
Females	*Blood group O*	18-30	56	95 (25)	Reference	Reference	Reference	Reference	Reference	Reference	Reference
		30-40	104	91 (27)	-4 (-13 to 5)	-4 (-13 to 5)	-5 (-14 to 4)	-5 (-14 to 5)	-3 (-12 to 6)	-3 (-12 to 7)	-3 (-13 to 6)
		40-50	165	93 (24)	-2 (-11 to 6)	-2 (-11 to 8)	-3 (-11 to 6)	-3 (-12 to 6)	-2 (-10 to 6)	0 (-9 to 9)	-2 (-11 to 7)
		50-60	232	103 (30)	8 (0 to 16)	7 (-1 to 16)	6 (-2 to 15)	7 (-2 to 15)	9 (1 to 17)	11 (2 to 20)	7 (-2 to 16)
		60-70	122	114 (31)	19 (10 to 28)	18 (8 to 27)	18 (9 to 27)	17 (8 to 27)	19 (10 to 28)	21 (12 to 30)	16 (6 to 26)
	Blood group non-O	18-30	58	116 (28)	Reference	Reference	Reference	Reference	Reference	Reference	Reference
		30-40	127	117 (33)	0 (-10 to 11)	0 (-11 to 10)	-1 (-12 to 10)	0 (-10 to 11)	1 (-10 to 12)	2 (-9 to 13)	0 (-12 to 11)
		40-50	201	118 (35)	2 (-8 to 12)	0 (-10 to 10)	0 (-11 to 10)	0 (-10 to 11)	2 (-8 to 12)	3 (-8 to 14)	-3 (-13 to 8)
		50-60	225	133 (35)	16 (6 to 26)	15 (5 to 25)	13 (3 to 24)	13 (3 to 23)	16 (6 to 26)	18 (7 to 29)	9 (-2 to 21)
		60-70	167	145 (39)	29 (19 to 40)	27 (16 to 37)	27 (16 to 38)	25 (14 to 35)	29 (19 to 39)	30 (18 to 41)	20 (8 to 33)

Supplementary table 4. Increase of FVIII:C as continuous variables with age (beta coefficients for decade are shown), stratified by sex and blood group, calculated in individuals age 40-70 years.

FVIII:C		Beta coefficient (95% CI) for decade (IU dL^{-1})						
		Unadjusted	**Adjusted for comorbidities**	**Adjusted for BMI**	**Adjusted for eGFR**	**Adjusted for CRP**	**Adjusted for hormone use**	**Adjusted for comorbidities, BMI, blood group eGFR, CRP, hormone use**
Males	*Blood group O*	10 (7 to 14)	10 (7 to 13)	10 (7 to 13)	8 (5 to 12)	9 (6 to 12)	-	7 (4 to 11)
	Blood group non-O	12 (8 to 17)	12 (7 to 16)	12 (8 to 17)	9 (5 to 14)	10 (6 to 15)	-	7 (2 to 12)
Females	*Blood group O*	12 (8 to 15)	11 (8 to 14)	11 (8 to 14)	11 (8 to 14)	11 (8 to 14)	11 (8 to 14)	9 (6 to 12)
	Blood group non-O	14 (10 to 17)	14 (10 to 17)	14 (10 to 18)	12 (8 to 16)	14 (10 to 17)	14 (10 to 17)	11 (7 to 15)