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Abstract: Hereditary disorders of primary haemostasis, characterized by mucocutaneous bleeding (MCB), are highly prevalent in children. Few cases are clearly monogenic but the overwhelming majority are classified as mild bleeding disorders, with wide clinical and laboratory heterogeneity suggestive of complex polygenic diseases. In this framework, and by homology with venous thrombosis, some functional polymorphisms affecting the haemostatic system should be considered. We evaluated the role of 18 common haemostatic polymorphisms on the occurrence and severity of MCB in a case-control study including 269 patients and 286 matched controls consecutively recruited. FV Leiden was associated with milder bleeding severity, assessed by a standardized bleeding score ($p=0.013$). Multivariate analysis revealed that three additional polymorphisms protected against MCB (F13 Leu34, $OR=0.66$, $95\%CI:0.47-0.94$, $p=0.024$; VKORC1 1173T, $OR=0.59$, $95\%CI:0.40-0.87$, $p=0.009$; and non-O blood group alleles, $OR=0.59$, $95\%CI:0.41-0.86$, $p=0.006$). When combined, these polymorphisms showed an additive protection ($OR=0.24$; $95\%CI:0.11-0.52$), supporting the polygenic nature of MCB. Our data suggest that some common polymorphisms affecting haemostasis-related genes could protect from bleeding.

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Study of 18 functional hemostatic polymorphisms in mucocutaneous bleeding disorders.

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Running head: Haemostatic SNPs in mucocutaneous bleeding

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Abstract

Hereditary disorders of primary haemostasis, characterized by mucocutaneous bleeding (MCB), are highly prevalent in children. Few cases are clearly monogenic but the overwhelming majority are classified as mild bleeding disorders, with wide clinical and laboratory heterogeneity suggestive of complex polygenic diseases. In this framework, and by homology with venous thrombosis, some functional polymorphisms affecting the haemostatic system should be considered. We evaluated the role of 18 common haemostatic polymorphisms on the occurrence and severity of MCB in a case-control study including 269 patients and 286 matched controls consecutively recruited. FV Leiden was associated with milder bleeding severity, assessed by a standardized bleeding score ($p=0.013$). Multivariate analysis revealed that three additional polymorphisms protected against MCB (*F13* Leu34, OR=0.66, 95%CI:0.47-0.94, $p=0.024$; VKORC1 1173T, OR=0.59, 95%CI:0.40-0.87, $p=0.009$; and non-O blood group alleles, OR=0.59, 95%CI:0.41-0.86, $p=0.006$). When combined, these polymorphisms showed an additive protection (OR=0.24; 95% CI:0.11-0.52), supporting the polygenic nature of MCB. Our data suggest that some common polymorphisms affecting haemostasis-related genes could protect from bleeding.

Word count: 163

Key words: mucocutaneous bleeding, mild bleeding disorders, gene polymorphism, thrombosis

Introduction

The haemostatic system has evolved in the animal kingdom to protect against fatal hemorrhages. A finely regulated interaction of platelets and haemostatic factors generates protective plugs preventing blood loss at sites of vascular injury. Disturbances in such interaction by structural anomalies or changes in levels of critical components of haemostasis are pathologically translated in either bleeding or thrombotic complications. Multiple gene mutations encoding natural inhibitors of the clotting process result in loss of plasma concentration/function of these proteins, i.e., antithrombin, proteins S or C, and are associated to high risk of venous thrombosis. The pathogenic predominance of these mutations in triggering thrombotic events supports, at least in some cases, that these single mutations have predominant contribution in the disease. However, the notion that venous thrombosis is a polygenic, complex disorder, gained support after identifying two common prothrombotic polymorphisms, factor V Leiden (FVL) and prothrombin G20210A [1, 2]. Since then, hundreds of studies have assessed the role of many polymorphisms as putative risk factors for thrombosis; however, the conclusions of many of them are discrepant [3-6].

Mucocutaneous bleeding (MCB) has high prevalence in children [7, 8]. A minority, constituting the more severe disorders, results from mutations affecting major platelet glycoproteins (i.e., GPIIb-IIIa or GPIb-V-IX) or plasma factors (i.e., complete absence of von Willebrand factor). In these cases, the symptoms can be explained almost exclusively by a single mutation determining defects in critical functions of platelet-vessel wall interaction. However, an overwhelming proportion of disorders manifested with MCB symptoms fall in the category of “mild bleeding disorders”, including most types of von Willebrand disease and platelet functional defects. In this category are also included more than 50% of all patients with MCB who remain undiagnosed after comprehensive laboratory studies [9]. These disorders are likely multifactorial, as is the case with most venous thrombosis [5, 6].

In this regard, the frequency of combined bleeding defects is greater than that predicted by chance [10], supporting and enlarging the notion initially raised by Sadler, who proposed that low VWF levels (and by extension some platelet function defects) should be considered risk factors rather than unique bleeding causes [11]. In a similar context, prothrombotic polymorphisms are reported to reduce the clinical severity of haemophilias [12]. Thus, it would be expected that functional haemostatic polymorphisms commonly regarded as procoagulants, modulate the clinical expression of bleeding disorders [13].

Now, we investigated the influence of 18 polymorphisms on the risk and bleeding severity in a case-control study involving 269 patients with MCB and 286 matched controls. These polymorphisms were selected by their relevant effect on different hemostatic proteins, as they affect the levels, function or aminoacid sequence, and all them have been extensively evaluated in different hemostatic diseases, mainly thrombotic disorders.

Materials and Methods

Patients

The Clinical Ethics Committee of the P. Catholic University of Chile approved this research. Blood samples were obtained after written informed consent, according to the Declaration of Helsinki. Consecutive patients (aged ≥ 4 years and ≤ 50 years), referred by physicians aware of the objectives of the study, were always interviewed by the same investigator using a standardized questionnaire [14], modified to assess mainly mucous and skin bleeding. The interviewer recorded the current and past bleeding episodes independently of the age at consultation. Briefly, the most frequent and typical symptoms (Table 1) were scored from 0 to 4, according to their frequency, duration, recurrence, and need and type of therapy. Other symptoms, of lesser frequency, less typical of primary haemostasis disorders, or those present only after exposure to risk,

were scored 0 (absent) or 1 (present). The recorded data were processed later providing a numerical assessment of bleeding severity (bleeding score, BS). Finally, the study included 269 patients (mean age 14.5 years, 60% female) who had pathologic bleeding. The controls, recruited independently of the patients with similar geographical, ethnic, social, economic and cultural background, were matched for age and sex (mean age 12.3 years, 55% female), and subjected to the same interview and exclusion criteria as the patients, selecting only non-bleeders (n= 253) or trivial bleeders (n= 24). Blood sampling processing, laboratory tests and diagnostic criteria for platelet function disorders, von Willebrand disease (VWD) and clotting factors deficiencies were described before [10]. Clinical characteristics, frequency of diagnosis and demographic data of patients and controls were previously reported [10]. Briefly, 39 patients (14.5%) had VWD, 50 (18.6%) had platelet dysfunction, 7 (2.6%) had coagulation factor deficiency and 13 (4.8%) had any combination of the previous three diagnosis. The remaining 160 patients (59.5%) had bleeding of unknown cause, classified in this category after a comprehensive laboratory work up.

Genotyping

Functional polymorphisms affecting the haemostatic system and methods of analysis are summarized in Table 2 [15-19]. The selection of these polymorphisms was carried out in terms of their functional effect and relevance reported in haemostatic diseases, mainly thrombotic disorders. Some of them have been previously shown to affect the plasma levels or functional effects of clotting factors or modify the bleeding severity in patients with hemorrhagic disorders.

We genotyped 18 polymorphisms affecting platelets; coagulation or fibrinolysis factors and others with haemostatic roles and ABO blood group. DNA was extracted by standard methods and Real Time PCR reactions were performed on a LC480 PCR

system (Roche). Direct sequencing was performed for randomly selected samples to confirm genotyping results. The ABO blood group was determined using standard agglutination techniques.

Statistical Analysis

Statistical analysis was performed using the Statistical Package for Social Science (version 15.0; SPSS, Chicago, IL, USA). Allele and genotype frequencies and deviations from Hardy-Weinberg expectations were calculated with the SNPstats software [20]. Continuous variables were tested for normal distribution by Kolmogorov-Smirnov's test. Data exhibiting normal or non-normal distribution are presented as mean $\pm$ standard deviation (SD) and as median and interquartile range (IQR), respectively. Categorical variables were compared by Pearson chi-squared test and continuous variables by Student's t-test or Mann-Whitney U-test, as appropriate. Multivariate analysis was used to study the independent contribution of polymorphisms with $p < 0.15$ in the univariate analysis. Multiple testing corrections were performed by the permutation test (number of permutations 10000). P values < 0.05 were considered statistically significant.

Results

Table 3 shows the genotype distribution of all studied polymorphisms in patients and controls. All polymorphisms included in our study followed the Hardy Weinberg equilibrium in the control group and, except for the ANV5 -1C/T polymorphism, when considering all subjects (data not shown).

Prevalence of FVL in the control group was almost thrice than that in patients group [$n = 9$ (3.1%) vs. $n = 3$ (1.1%), respectively] but this difference was not statistically significant ($p = 0.09$) (Table 3). However, the 3 patients with FVL allele had

significantly lower BS median= 4, (IQR: 2-4) than the 266 patients without the mutation
BS median= 7, (IQR: 5-11, $p= 0.013$).

None of the other polymorphisms was significantly associated with the BS, both in the
whole group of 269 patients and in the cohort of 160 patients with bleeding from
unknown cause (data not shown).

Out of the 18 analyzed polymorphisms, three of them (*F13* Val34Leu, *VKORC1*
C1173T and Non-O blood type) were significantly more frequent in the control non-
bleeding population than in patients suggesting a bleeding protection. In contrast,
carriers of the T allele of the *F12* -4 C>T polymorphism were significantly less
frequent in controls than in patients, suggesting an associated hemorrhagic risk for this
allele (Table 3).

When the analysis was restricted to the 160 patients without a final diagnosis after a
complete laboratory work-up, the results were essentially the same, excepting the lack
of statistical significance of ABO blood group as compared with the controls (Table 3).

Multivariate analysis including polymorphisms with $p < 0.15$ in the univariate analysis
revealed that only *F13* Val34Leu, *VKORC1* C1173T and ABO polymorphisms
maintained their condition of bleeding protection (OR= 0.66, 95%CI: 0.47-0.94,
 $p=0.024$; OR= 0.59, 95%CI: 0.40-0.87, $p= 0.009$; and OR= 0.59, 95%CI: 0.41-0.86, $p=$
0.006, respectively). Identical statistical testing in the patients with unknown diagnosis
gave similar results to the univariate analysis.

Multiple testing adjusting by permutation analysis (number of permutations 10,000), did
not modify these results (data not shown).

In a framework of a polygenic disease, we evaluated the effect in MCB for the
combination of the three polymorphisms that seems to have a role in this disease after
the multivariate analysis. The profile with homozygous genotypes of the common allele
in the general population for these polymorphisms (*F13* Val34Val + *VKORC1* C1173C
+Blood group O) was taken as the reference category (OR= 1) to calculate the OR that

confers all alternative combinations of these three SNPs. As shown in Table 4 the combination of these polymorphisms had an additive protective effect for bleeding risk, which is maximal with the concurrency of the three polymorphisms in the same individual.

Discussion

The balance between procoagulant and anticoagulant factors is finely tuned to ensure an appropriate, timely and spatially restricted haemostatic response, avoiding its extension beyond the limits of vascular injury and deriving in blood flow obstruction (thrombus formation). In such scenario, it is well known that hypo- or hyperfunction of a singular clotting factor may be causally related to bleeding or thrombosis, respectively. The overwhelming majority of such studies have addressed the risk of thrombosis, but not of bleeding associated with such polymorphisms. The modulating role of common prothrombotic mutations on the bleeding phenotype in severe congenital bleeding disorders [12, 21] or in acquired conditions with high bleeding risk or complications [22, 23] have been reported. However, such type of studies are lacking in patients with mild bleeding disorders, clinically characterized by MCB.

Although the ABO system is not an inherent haemostatic polymorphisms, individuals with the O blood group have significantly lower plasma levels of FVIII and VWF than those with non-O blood groups (A, B and AB) [24, 25]. In our study, we found that O genotype was significantly over-represented in patients as compared with controls. A trend in the same direction was observed in the 160 patients with no etiologic diagnosis. These results support previous ones showing that the O blood group increases the bleeding risk [26] and protects against both venous [27] and arterial thrombosis [28, 29]. In the same line, our results showed that the non-O blood group exerts a weak protection from bleeding (OR: 0.63 95% CI: 0.44-0.89).

We also evaluated the protective role on MCB of other functional haemostatic polymorphisms with a more controversial pro-thrombotic role. We observed that *F13* Leu34 and *VKORC1* 1173T alleles, whose relationship with thrombosis is still unresolved [30-32] were significantly over-represented in controls than in the whole patient population, as well as in the cohort of patients with unknown diagnosis. Accordingly, these polymorphisms would decrease the hemorrhagic risk. In contrast, the *F12* -4T polymorphic allele, also considered a controversial thrombotic risk factor [33, 34] was more prevalent in patients than in controls, favoring the bleeding tendency (Table 3).

The multivariate analysis, which incorporated only SNPs with $p < 0.15$ in the univariate analysis, showed that only 3 out of the 18 polymorphisms (*F13* Val34Leu, *VKORC1* C1173T and ABO blood system) were protective for bleeding.

Currently, our incomplete pathophysiological understanding of bleeding in most patients with skin and mucous hemorrhages precludes the use of specific diagnostic tests and explains why more than 50% of them end up without a known diagnosis [9]. In this regard, most mild bleeding disorders are likely multi-factorial, with symptoms emerging only after the accretion of less severe hereditary or acquired bleeding risk factors, as demonstrated in venous thrombosis [35]. This notion is founded in several pieces of evidence: A. Combined mild bleeding disorders (i.e., VWD + platelet function defect) are more frequent (11.5%) than predicted by chance [10]. B. There is a weak or null correlation of bleeding severity with some laboratory findings, e.g., plasma VWF [10, 11, 35] or most platelet aggregation defects [10]. C. Acquired conditions (i.e., intake of aspirin) trigger bleeding episodes in up to 44% of patients with oligo-symptomatic hereditary MCB, whereas it seldom induces hemorrhages in healthy subjects. In this regard, the aspirin-induced platelet aggregation and secretion defects in non-bleeder individuals are usually more severe than those of aspirin-free patients diagnosed with platelet secretion disorders [10].

This polygenic conception of mild bleeding disorders is supported by the results shown in Table 4. The reference combination of the 3 polymorphisms identified in the multivariate analysis (*F13* Val34Val + *VKORC1* C1173C + Blood group O) was present in a patient/control ratio of 1.5. In contrast, the profile with the combination of our guessed protective alleles (*F13* Leu34 + *VKORC1* 1173T + Blood group Non-O) were found in a patient/control ratio of 0.37 in the whole sample (OR: 0.24; 95%CI: 0.11-0.52). This protection was slightly increased in bleeders of unknown cause (OR: 0.21; 95%CI: 0.08-0.52).

The above results strongly support the notion that MCB behaves like a complex disease, similar to our current concept of thrombotic events. Just a minority of the inherited mucocutaneous bleeding disorders have a strong monogenic component (i.e., Glanzmann's thrombasthenia, Bernard-Soulier syndrome) while most of them result from complex combinations of various risk factors whose final effect is tilting the haemostatic balance toward bleeding. Here we showed how different and common functional haemostatic polymorphisms, so far visualized mainly as thrombotic risk factors, may also modulate the bleeding risk. The polygenic conception of MCB is further supported by the additive or synergistic bleeding risks resulting from combinations of polymorphisms in the same patient. Our study does not allow concluding that the studied polymorphisms, even when combined, have enough power to explain *per se* the bleeding symptoms, but our results do support their modulating role in either preventing or enhancing the bleeding in patients with stronger risk factors, such as low plasma VWF or platelet dysfunctions. In this context, the large clinical heterogeneity of VWD and platelet function diseases may be partially explained by the enhancing or inhibitory role of these polymorphisms.

Our study has some limitations that warrant our attention. First, the choice of SNPs is arbitrary, and other potentially relevant SNPs could be added. We took a hypothesis-driven candidate gene approach rather than a whole genome analysis scan (GWAS) –

based approach because it was clear that the number of events to be analyzed would be small, and we were intended to identify pertinent functional loci variants with moderate to large functional effect. Future GWAS and sequencing approaches may add relevant variants in other haemostatic elements and unknown pathways that could play a role in MCB. Second, the fact that FXIII and VKORC1 polymorphisms did not follow the HWE in the patient sample questions the suggested protective effect on MCB. Finally, a more stringent multiple testing analysis revealed that only the ABO blood group maintained its significant effect on MCB. Therefore, further studies with more patients and controls are required to confirm these results.

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TABLE 1. Site, frequency of bleeding and family history in patients with mucocutaneous bleeding and controls.

Symptoms	Frequency %		
	Controls n=286	All patient population n=269	Patients with bleeding of unknown cause n=160
Menorrhagia	1.4(70)*	90.0(90)*	87.8(49)*
Ecchymoses	19.6	78.8	79.4
Epistaxis	24.1	77.3	78.1
Cautery	1.0	24.2	25.6
Bleeding after dental extractions	0.0(115)*	66.0(103)*	59.6(57)*
Gum bleeding	12.6	53.9	53.1
Post-partum bleeding	0.0(8)*	51.7(29)*	64.7(17)*
Bleeding after minor injuries	1.4	49.4	46.9
Surgical bleeding	0.0(77)*	48.0(102)*	44.6(65)*
Hematomas (mostly superficial)	3.1	34.2	26.9
Bleeding after aspirin intake	0.0(177)*	31.4(102)*	26.1(69)*
Bleeding requiring transfusion	0.0	23.4	16.9
Hemoptoic sputum	0.3	22.3	21.9
Petechiae	0.7	16.0	16.3
Occult blood in stools	1.0	14.9	15.6
Tongue frenulum bleeding	0.0	9.7	9.4
Gastrointestinal bleeding	0.7	7.8	6.3
Umbilical bleeding	0.0	7.4	9.4
Otorrhagia	0.0	4.5	3.1
Hemarthroses	0.0	4.5	3.8
Deep hematomas	0.0	0.7	0.0
Hematuria	0.0	3.3	3.1
Hemoptyses	0.0	0.0	0.0
Family history of bleeding:			
-First degree relatives	23.8	28.6	33.1
-Second degree relatives	15.7	16.4	16.3
-First and second degree relatives	11.9	42.4	35.6
Total	51.4	87.4	85.0

* Values in parentheses reflect the number of individual exposed to risk.

TABLE 2. Polymorphisms evaluated and method of genotyping.

Gen symbol	dbSNP	Change	Other name	Genotyping method
<i>ITGA2</i>	rs1126643	C/T (F253)	GPIa C807T	Taqman SNP Genotyping Assay C__2390370_50 (Applied Biosystems)
<i>ADRA2A</i>	rs553668	A/G (3'UTR)	ADRA2A G1780A	Taqman SNP Genotyping Assay C__996424_20 (Applied Biosystems)
<i>TUBB1</i>	rs463312	A/C (Q43P)	TUBB Q43P	PCR-ASRA (15)
<i>ITGB3</i>	rs5918	T/C (L59P)	GPIIIa HPA-1	FRET probe (16)
<i>GPIBA</i>	rs6065	C/T (T161M)	GPIb α HPA-2	FRET probe (16)
<i>GPIBA</i>	VNTR	23 bp	GPIb α VNTR	Capillar electrophoresis Primer F 5'(Hex) CTACTGAACCAACCCCAAGC 3' Primer R 5' CTTGTGGCAGACACCAGGAT 3'
<i>F2</i>	rs1799963	G/A (3'UTR)	PT G20210A	Taqman SNP Genotyping Assay C__872602_20 (Applied Biosystems)
<i>F5</i>	rs6025	G/A (R534Q)	FV Leiden	Taqman SNP Genotyping Assay C__11975250_10 (Applied Biosystems)
<i>F7</i>	rs36208070	10 bp	<i>F7</i> -323 ins/del	Capillar electrophoresis Primer F 5'(Fam) GGTCTGGAGGCTCTCTTCAA3' Primer R 5'AGAGCGGACGGTTTTGT3'
<i>F12</i>	rs1801020	C/T (5'UTR)	<i>F12</i> -4 C>T	Taqman SNP Genotyping Assay C__1989313_20 (Applied Biosystems)
<i>F13</i>	rs5985	G/T (V34L)	<i>F13</i> Val34Leu	FRET probe (17)
<i>SERPINE1</i>	rs1799889	4G/5G (5'UTR)	<i>PAI-1</i> 4G/5G	FRET probe (18)
<i>SERPINE10</i>	rs2232708	G/A (G271S)	<i>ZPI</i> G250S	FRET probe Primer F: 5'CCCATTGACCCTGTCTTC3' Primer R: 5'CAAAGGTGGAGCCAAACT3' Sensor 5'CACCTGGACAAGTACAAGACCATTAAAGGT-FI 3' Anchor 5'(Red640)CCCATGATGTACAGTGCAGG-P 3'
<i>ANXA5</i>	rs11577945	G/A (5'UTR)	ANV -1 C/T	Taqman SNP Genotyping Assay C__25471553_10 (Applied Biosystems)
<i>VKORC1</i>	rs9934438	C/T (intron)	<i>VKORC1</i> C1173T	FRET probe Primer F: 5'GGATAGGGTCAGTGACATGGAAT 3' Primer R: 5'GCCCCGAGAAAGGTGATT3' Sensor 5'CTAGTCCAAGAGTCGATGATCTCC- FI 3' Anchor 5'(Red640)GGCACC GGACCTTTGGCC-P 3'
<i>HAPB2</i>	rs7080536	G/A (G534E)	<i>FSAP</i>	FRET probe (19)
<i>SELPLG</i>	VNTR	36 bp	<i>PSGL-1</i>	Capillar electrophoresis Primer F 5' (Fam) TCCACGGATTTCAGCAGCTAT3' Primer R 5'GGGAATGCCCTTGTGAGTAA3'

ITGA2: integrin, alpha 2; GPIa: platelet glycoprotein Ia; *ADRA2A*: adrenergic alpha-2A receptor; *TUBB1*: tubulin, beta 1; *ITGB3*: integrin beta 3; GPIIIa: platelet glycoprotein IIIa; HPA-1: human platelet antigen 1; GPIBA: platelet glycoprotein Ib, alpha polypeptide; HPA-2: human platelet antigen 2; *F2*: coagulation factor II (prothrombin); *F5*: coagulation factor V; *F7*: coagulation factor VII; *F12*: coagulation factor XII; *F13*: coagulation factor XIII; ANXA5: annexin V; *PAI-1*: plasmin activator inhibitor-1; *VKORC1*: vitamin K epoxide reductase complex; *ZPI*: protein Z-related protease inhibitor, *HAPB2*: hyaluronan binding protein 2; *FSAP*: factor VII-activating protease; *SELPG*: selectin P ligand; *PSGL-1*: P selectin glycoprotein ligand-1.

TABLE 3. Distribution of genotypes for each polymorphism in patients and controls.

Genotype		ALL Patients n=269	p value# OR (95%CI)	UC * Patients n=160	p value# OR (95%CI)	Controls n=286
GPIa C807T	CC	87 (32.3%)	0.90 1.02 (0.72-1.46)	55 (34.4%)	0.75 0.93 (0.62-1.41)	94 (32.9%)
	CT	130 (48.3%)		77 (48.1%)		145 (50.7%)
	TT	52 (19.3%)		28 (17.5%)		47 (16.4%)
ADRA2A G1780A	GG	113 (42.0%)	0.06 1.38 (0.99-1.93)	69 (43.1%)	0.16 1.32 (0.89-1.95)	143 (50.0%)
	GA	124 (46.1%)		67 (41.9%)		124 (43.4%)
	AA	32 (11.9%)		24 (15.0%)		19 (6.6%)
TUBB1 Q43P	QQ	221 (82.5%)	0.19 0.75 (0.49-1.15)	132 (82.5%)	0.29 0.77 (0.47-1.26)	221 (78.4%)
	QP	45 (16.8%)		26 (16.2%)		54 (19.1%)
	PP	2 (0.8%)		2 (1.2%)		7 (2.5%)
GPIIIa HPA1	TT	207 (77.0%)	0.16 1.35 (0.89-2.04)	127 (79.4%)	0.53 1.17 (0.72-1.90)	234 (81.8%)
	TC	58 (21.6%)		31 (19.4%)		49 (17.1%)
	CC	4 (1.5%)		2 (1.2%)		3 (1.1%)
GPIIb HPA2	CC	203 (76.3%)	0.96 1.01 (0.68-1.50)	122 (76.7%)	0.95 0.99 (0.62-1.56)	218 (76.5%)
	CT	62 (23.3%)		37 (23.3%)		58 (20.4%)
	TT	1 (0.4%)		0 (0%)		9 (3.2%)
GPIIb VNTR	CC+CD+DD	213 (79.2%)	0.59 1.12 (0.74-1.71)	126 (78.25%)	0.57 1.15 (0.71-1.86)	226 (81.0%)
	BB+BD+CB	56 (20.8%)		34 (21.25%)		53 (19.0%)
PT G20210A	GG	264 (98.1%)	0.63 0.75 (0.24-2.41)	158 (98.8%)	0.37 0.5 (0.1-2.46)	279 (97.5%)
	GA	5 (1.9%)		2 (1.2%)		7 (2.5%)
FV Leiden	GG	266 (98.9%)	0.09 0.35 (0.09-1.30)	159 (99.4%)	0.06 0.19 (0.02-1.54)	277 (96.8%)
	GA	3 (1.1%)		1 (0.6%)		9 (3.1%)
F7 -323ins/del	AA	198 (73.6%)	0.08 0.72 (0.50-1.04)	118 (73.8%)	0.12 0.72 (0.47-1.1)	191 (66.8%)
	AB	66 (24.5%)		40 (25.0%)		87 (30.4%)
	BB	5 (1.9%)		2 (1.2%)		8 (2.8%)
F12 -4C>T	CC	103 (38.3%)	0.03 1.46 (1.04-2.05)	58 (36.2%)	0.02 1.59 (1.07-2.37)	136 (47.5%)
	CT	133 (49.4%)		79 (49.4%)		123 (43.0%)
	TT	33 (12.3%)		23 (14.4%)		27 (9.4%)
F13 Val34Leu	GG	147 (54.9%)	0.005 0.62 (0.44-0.87)	90 (56.6%)	0.005 0.58 (0.39-0.86)	123 (43.0%)
	GT	93 (34.7%)		51 (32.1%)		137 (47.9%)
	TT	28 (10.4%)		18 (11.3%)		26 (9.1%)
PAI-1 4G/5G	5G5G	95 (35.3%)	0.80 0.95 (0.67-1.35)	57 (35.6%)	0.7 0.94 (0.63-1.41)	98 (34.3%)
	4G5G	126 (46.8%)		75 (46.9%)		132 (46.1%)
	4G4G	48 (17.8%)		28 (17.5%)		56 (19.6%)
ZPI G250S	GG	264 (98.1%)	0.84 0.88 (0.27-2.92)	157 (98.1%)	0.87 0.89 (0.22-3.60)	279 (97.9%)
	GA	5 (1.9%)		3 (1.9%)		6 (2.1%)
ANX 5 -1C/T	CC	222 (82.5%)	0.29 0.80 (0.52-1.22)	128 (80.0%)	0.81 0.94 (0.58-1.52)	226 (79.0%)
	CT	47 (17.5%)		32 (20.0%)		59 (20.6%)
	TT	0 (0%)		0 (0%)		1 (0.4%)
VKORC1 C1173T	CC	93 (34.7%)	0.013 0.63 (0.43-0.91)	60 (37.7%)	0.005 0.55 (0.36-0.84)	71 (25.0%)
	CT	113 (42.2%)		66 (41.5%)		144 (50.7%)
	TT	62 (23.1%)		33 (20.8%)		69 (24.3%)
FSAP G534E	GG	264 (98.1%)	0.82 0.87 (0.26-2.89)	155 (96.9%)	0.52 1.48 (0.45-4.94)	276 (97.9%)
	GA	5 (1.9%)		5 (3.1%)		6 (2.1%)
PSGL1 VNTR	AA	195 (72.5%)	0.84 1.04 (0.71-1.52)	108 (67.5%)	0.19 1.32 (0.86-2.03)	211 (73.8%)
	AB+AC+BB	72 (27.5%)		51 (32.5%)		75 (26.2%)
ABO	O	184 (68.4%)	0.009 0.63 (0.44-0.89)	106 (66.2%)	0.074 0.69 (0.46-1.04)	165 (57.7%)
	No-O	85 (31.6%)		54 (33.8%)		121 (42.3%)

* UC: Unknown Cause.

OR were calculated taking as reference category the most frequent homozygous genotypes of each polymorphism in the general population or combination of genotypes for the GPIIb VNTR.

TABLE 4. Additive effect of combination of polymorphisms on the risk of developing MCB.

Polymorphisms combinations [#]	All Patients n=269	<i>p</i> value OR (95% CI)	UC* Patients n=160	<i>p</i> value OR (95% CI)	Controls n=286
<i>F13</i> Val34Val & <i>VKORC1</i> C1173C & O ABO	33	Reference 1	22	Reference 1	22
<i>F13</i> Leu34 & <i>VKORC1</i> 1173T	82	0.002 0.44 (0.24-0.81)	43	0.001 0.34 (0.17-0.69)	123
<i>F13</i> Leu34 & no-O ABO	37	0.004 0.36 (0.18-0.72)	24	0.034 0.35 (0.1-0.76)	67
<i>VKORC1</i> 1173T & no-O ABO	46	<0.001 0.32 (0.17-0.62)	29	0.001 0.30 (0.14-0.63)	94
<i>F13</i> Leu34 & <i>VKORC1</i> 1173T & no-O ABO	19	<0.001 0.24 (0.11-0.52)	11	0.001 0.21 (0.08-0.52)	51

* UC: Unknown Cause.

[#] *F13* Leu34 includes *F13* Val34Leu and *F13* Leu34Leu genotypes.

VKORC1 1173T includes *VKORC1* C1173T and *VKORC1* T1173T genotypes.

**UNIVERSIDAD
DE MURCIA**

**DEPARTAMENTO DE
MEDICINA INTERNA**
Facultad de Medicina

Murcia, 10th May 2010,

Dear Dr. Ganser,

Thank you for considering our manuscript “Mucocutaneous bleeding disorders: role on development and severity of bleeding of 18 common hemostatic polymorphisms”, by **A.I. Antón et al.**, potentially publishable in Annals of Hematology.

We feel really grateful to all the reviewers and have tried to answer all their questions and meet all their demands. Accordingly, we would like you to re-consider this revised version of the manuscript for publication in Annals of Hematology.

The point-by-point explanation of changes made to the original manuscript ensue within what the next following pages.

We hope that all comments and corrections satisfy the reviewers criticisms and that now our manuscript be accepted for publication in this journal..

We look forwards to hearing from you soon.

Yours sincerely,

Prof. V Vicente.

Reviewer 1:

In their study entitled "Mucocutaneous bleeding disorders: role on development and severity of bleeding of 18 common haemostatic polymorphisms." Antón et al analyzed arbitrarily selected known polymorphisms in genes of procoagulatory and anticoagulatory proteins for an association with mucocutaneous bleeding. They tested 269 patients and 286 controls in a case control study design. The authors identified three components (non-O blood group, F13 Leu34 and VKORC1 1173T) to protect from mucocutaneous bleeding in a statistically significant fashion.

The study is interesting, and the conclusion that mild bleeding disorders are not caused by genetic defects in one gene but are polygenic and multifactorial similar to thrombotic disorders, is plausible. However, besides the arbitrary selection of the 18 SNPs the study has several major limitations that have to be addressed:

Only 160 patients had unexplained bleeding disorders, in 109 patients platelet defects, vWD or coagulation factor deficiencies were identified. Thus, the number of patients with unexplained bleeds was small and the study has to be considered a hypothesis finding study. The authors have to make this very clear (eg. in the title).

We acknowledge the appropriate comments from reviewer 1. We agree that this study is an hypothesis finding study and the title was modified accordingly. Moreover, we have moderated our conclusions both in the Abstract and Discussion sections.

Additionally, we have explained the selection of the studied polymorphisms in the introduction section. These 18 polymorphisms have a relevant effect on different hemostatic proteins, as they affect the levels, function or aminoacid sequence, and all have been evaluated in different hemostatic diseases, mainly thrombotic disorders.

Certainly, other polymorphisms of the haemostatic system could follow this criteria, but

with more controversial effects, low prevalence or less number of studies supporting their functional role in thrombosis.

The authors have to test the distribution of their genotypes for Hardy Weinberg equilibrium. Clearly, in the patient population the FXIII polymorphism significantly deviates from the Hardy Weinberg distribution ($p= 0.0149$). Possibly, technical problems or a selection bias is responsible for this deviation. Undoubtedly, data that deviate from Hardy Weinberg cannot be used for statistical calculations! The distribution for VKORC1 is also borderline in the patients!

We acknowledge and understand the reviewer's concerns about deviations of the Hardy Weinberg equilibrium (HWE) in our study. Potential technical problem of genotyping were discarded by consistent results between genotyping and sequencing analysis performed in random samples for all polymorphisms. There are several explanations why the observed genotype frequencies may deviate significantly from those expected by HW law and why genotypes have different likelihoods of being included, even when determination of genotype was methodologically correct. One or more of the assumptions of the model might be incorrect, non-random mating (inbreeding or an allele effect on the mating) or gene flow may have occurred, or selection operated (Sham P. Statistics in human genetics. New York, NY: Oxford University Press, 1998). There could also be an error at sampling: the studied population is not well defined or the sample size may be too small. If the genotype distribution in the control population misses the HWE, the results should be treated cautiously because the observed genotype distribution does not represent the genotype distribution in healthy (non-diseased) people and, therefore, conclusions cannot be drawn for the significance of the

investigated polymorphism. All polymorphisms included in our study followed the HWE in the control group and, except for the *ANV5* –1C/T, when considering all subjects (Table A). This statement has been included in the revised manuscript (line 19, page 6). However, relationship between genotype and disease might occur if the genotype distribution in the investigated (diseased) population does not fulfill the HWE law while the healthy reference population does it (Lee WC. Searching for disease-susceptibility loci by testing for Hardy-Weinberg disequilibrium in gene bank of affected individuals. American Journal of Epidemiology; 2003 (5):397-400). Nevertheless, we agree with reviewer 1 that deviation of *F13* and *VKORC1* polymorphisms of patient population from the HWE questions the role of these two polymorphisms in MCB. This limitation has also been included in the revised manuscript (line 5, page 11).

Table A. Exact test for Hardy Weinberg equilibrium for all studied polymorphisms

	Patients	Controls	All subjects
GPIa C807T	0.8	0.54	0.79
ADRA2A G1780A	0.89	0.31	0.43
TUBB1 Q43P	1	0.15	0.26
GPIIIa HPA1	1	0.73	0.83
GPIIbα HPA2	0.14	0.066	0.7
GPIIbα VNTR	*	*	*
PT G20210A	1	1	1
FV Leiden	1	1	1
F7 -323ins/del	1	0.69	0.75
F12 -4C>T	0.36	1	0.51
F13 Val34Leu	0.032	0.18	0.62
PAI-1 4G/5G	0.61	0.34	0.3
ZPI G250S	1	1	1
ANX 5 -1C/T	0.24	0.22	0.048
VKORC1 C1173T	0.019	0.91	0.12
FSAP G534E	1	1	1
PSGL1 VNTR	*	*	*
ABO	*	*	*

* HWE not determined for multiallelic polymorphisms.

The performance of functional assays e.g. for FXIII, FVIII or FXII would be highly valuable to proof that carriers of the SNPs or non-O blood group had indeed lower or higher coagulation factor levels, respectively.

The functional effect of FXIII, FVIII or FXII polymorphisms have been deeply investigated and evidenced elsewhere, also by our group. Moreover, in this study, we

have also demonstrated the effect of *F12* -4 C>T polymorphism and O blood group on plasma levels of hemostatic factors. As shown in Table B, carriers of *F12* -4T allele had significantly lower levels of FXII in plasma than carriers of the C allele. Similarly, O blood group carriers had lower levels of FVIII and VWF than carriers of no-O blood type. Unfortunately, we have no data for FXIIIc levels or activity, but others studies have demonstrated the functional effect of the *F13* V34L polymorphism (Ariëns RA, Philippou H, Nagaswami C, Weisel JW, Lane DA, Grant PJ. The factor XIII V34L polymorphism accelerates thrombin activation of factor XIII influencing the stabilization of the fibrin clot (Blood. 2000 Aug 1;96(3):988-95).

TABLE B. Functional effect of *F12* and ABO polymorphisms on FXII, FVIII and VWF levels in plasma. Values represented the % of the value observed in a pool of 100 healthy blood donors.

<i>F12</i> -4 C>T	N	FXII	
CC	238	109.34 ± 20.72	
CT	256	90.72 ± 20.74	
TT	60	60.57 ± 16.24 p< 0.0001	
ABO blood type	N	FVIII	VWF
O Group	349	78.77 ± 26.10	72.40 ± 32.54
Non-O Group	206	94.37 ± 29.22 p < 0.0001	101.48 ± 39.94 p< 0.0001

The authors should discuss why they believe that VKORC1 1173T confers a protection from bleeding.

It has been widely reported that the VKORC1 haplotypes regulate the transcription rate of VKORC1. Thus, VKORC1 polymorphisms influence the anticoagulant therapy and the risk of bleeding associated with this treatment. The C1173T variant is in high linkage disequilibrium with -1639G>A polymorphisms ($r^2=0.946$), and both have been considered as good markers of interindividual variability in VKORC1 activity (Wang D et al., Regulatory polymorphisms in vitamin K epoxide reductase complex subunit 1 (VKORC1) affects gene expression and warfarin dose requirements. Blood 2008; 112:1013). Carriers of 1173T or -1639A alleles have a lower mRNA VKORC1 and hence, they need a lower coumarin dose for anticoagulation treatment.

The potential role of this polymorphism on the haemostatic system without oral anticoagulation has been investigated without clear consensus. The available data about VKORC1 genotype and risk/protection from thrombosis or bleeding are nowadays conflictive. Thus, a lower risk of VKORC1 genotype on large-artery atherosclerotic stroke have been recently described (Shyu HY et al. Genotype polymorphisms of GGCX, NQO1, and VKORC1 genes associated with risk susceptibility in patients with large-artery atherosclerotic stroke. Clin Chim Acta, 2010; 411:840) whereas others authors fail to find such association in other thrombotic pathologies (Arnold ML et al. Single nucleotide polymorphisms in the VKORC1 gene and the risk of stroke in the Southern German population. Thromb Haemost 2008, 100:614; Lemmens, J et al. Lack of association between variants in the VKORC1 gene and cerebrovascular or coronary heart disease (Thromb Haemost 2008; 6:2220; Smadja DM et al.). No clear link between VKORC1 genetic polymorphism and the risk of venous thrombosis or

peripheral arterial disease. Thromb Haemost 2008; 99:970; Lacut K et al. Vitamin K epoxide reductase genetic polymorphism is associated with venous thromboembolism: results from the EDITH Study. J Thromb Haemost 2007; 5:2020).

In this framework, we considered of interest to evaluate the role of VKORC1 genotype on bleeding risk. Certainly, our findings suggesting that VKORC1 1173T confers a mild protection from bleeding lack a pathophysiological explanation and require further confirmation.

No correction for multiple testing was performed, the authors have to explain why they did not do that and thus cannot exclude statistical associations due to chance.

We appreciate the comment from reviewer 1. Multiple testing corrections adjust p-values derived from multiple statistical test to correct for occurrence of false positives. This correction is required for massive microarray data analysis, meta-analysis or genome wide association studies, where thousands of genes or SNPs are analyzed simultaneously in different conditions. Our study included only 18 polymorphisms, however, we agree with reviewer 1 than correction for multiple testing would be desirable. Therefore, we performed this analysis by the permutation test (number of permutations: 10,000, $p < 0.05$). As indicated in the revised manuscript (line 9, page 7), all polymorphisms maintained the statistical significance. P-values corrected according to permutation test (PT) are shown in table C.

Table C. Crude and multiple testing adjustment by permutation test (with 10000 permutations) for differences in polymorphisms between patients and controls.

	Crude <i>p-value</i>	Adjusted <i>p-value (PT)</i>
<i>F12</i> -4C>T	0.030	0.024
<i>F13</i> Val34Leu	0.005	0.004
<i>VKORC1</i> C1173T	0.013	0.023
ABO	0.009	0.008

However, we admit that more stringent multiple testing analysis (Bonferroni or Benjamini and Hochberg adjustments) reveals that only the ABO blood group maintained its significant effect on MCB. This limitation has also been described in the revised manuscript (line 7, page 11).

Reviewer 2:

Reviewer #2: Please add a more detailed description of the employed criteria and the whole process of selection of the tested SNPs.

We tested these 18 polymorphisms because they have a relevant functional effect on the affected protein and all have been evaluated in different hemostatic diseases, mainly thrombotic disorders. Certainly, other polymorphisms of the haemostatic system could follow this criteria, but with more controversial effects, low prevalence or less number of studies supporting their functional or pro-thrombotic effect. This is specifically indicated in line 11, page 4 of the revised manuscript. Moreover, not-directed analysis could also have been performed, but due to economical cost, size and heterogeneity of the case-control study, this approach was discarded.

Were other haemostasis associated SNPs genotyped in the present sample, but not included in this publication?

We have recently published a manuscript evaluating the effect on the platelet function of 6 polymorphisms affecting platelet proteins in the same patient and control populations (Martínez C, Antón AI, Corral J, Quiroga T, Panes O, Lozano ML, González-Conejero R, Teruel R, Navarro-Núñez L, Pereira J, Mezzano D, Vicente V, Rivera J. Genotype-phenotype relationship for six common polymorphisms in genes affecting platelet function from 286 healthy subjects and 160 patients with mucocutaneous bleeding of unknown cause. Br J Haematol. 2009 Jun; 146(1):95-103), but no additional polymorphisms were evaluated in this case-control study.

How do you explain the lack of association of the clinical severity of bleeding (measured as the bleeding score) with the load of alleles determined to be risk-factors for MCB?

This is an interesting question. The explanation may be similar to that described for thrombosis. The polymorphism may increase the risk to develop an event but it is not necessarily related to the severity of the event. Only FV Leiden, which is in our opinion the strongest polymorphism on the hemostatic system may have both functions, e.g. protection against mucocutaneous bleeding (the low prevalence of this polymorphism did not allow to get statistical significance in our study) and reduction in the severity of the bleeding.

Discussion on FV Leiden is a bit too detailed, considering the low case number, and the not statistical significant results.

We agree with reviewer 2. This is new evidence supporting the differential effect of FV Leiden in thrombosis and hemorrhage, but certainly our results were not statistically significant due to the low case number. Accordingly and following the suggestion of reviewer 1, we deleted this discussion.

Page 6 line 45: Table 3 does not give the bleeding score

We apologize for the mistake, which has been corrected in the revised manuscript.