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ORIGINAL ARTICLE

Functional variation in the arginine vasopressin 2 receptor as a modifier of human plasma von Willebrand factor levels

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Summary. *Objectives:* Stimulation of arginine vasopressin 2 receptor (V2R) with arginine vasopressin (AVP) results in a rise in von Willebrand factor (VWF) and factor VIII plasma levels. We hypothesized that gain-of-function variations in the V2R gene (*AVPR2*) would lead to higher plasma levels of VWF and FVIII. *Methods and Results:* We genotyped the control populations of two population-based studies for four *AVPR2* variations: a-245c, G12E, L309L, and S331S. Rare alleles of a-245c, G12E, and S331S, which were in linkage disequilibrium, were associated with higher VWF propeptide, VWF and FVIII levels. The functionality of the G12E variant was studied in stably transfected MDCKII cells, expressing constructs of either 12G-V2R or 12E-V2R. Both V2R variants were fully glycosylated and expressed on the basolateral membrane. The binding affinity of V2R for AVP was increased three-fold in 12E-V2R–green fluorescent protein (GFP) cells, which is in accordance with increased levels of VWF propeptide associated with the 12E variant. The dissociation constant (K_D) was 4.5 nM [95% confidence interval (CI) 3.6–5.4] for 12E-V2R–GFP and 16.5 nM (95% CI 10.1–22.9) for 12G-V2R–GFP. AVP-induced cAMP generation was enhanced in 12E-V2R–GFP cells. *Conclusions:* The 12E-V2R variant has increased binding affinity for AVP, resulting in increased signal transduction, and is associated with increased levels of VWF propeptide, VWF, and FVIII.

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Introduction

The mechanisms underlying interindividual variations in von Willebrand factor (VWF) and coagulation factor VIII plasma levels are poorly understood. VWF is the carrier protein of FVIII, and their levels therefore usually fluctuate together. There are indications that VWF and FVIII levels are, in part, determined genetically [1–3]. Besides ABO blood group and variations in the genes encoding VWF and FVIII, genetic variations in proteins involved in regulation of the plasma levels of VWF and FVIII may contribute to variations in VWF and FVIII levels.

A candidate modifier of VWF and FVIII levels is the arginine vasopressin 2 receptor (V2R). The main function of V2R is to maintain blood volume and blood pressure by stimulating water retention in the kidney. Upon binding of arginine vasopressin (AVP) to V2R in renal principal cells, a cAMP signaling cascade is initiated, resulting in increased expression of the water channel aquaporin-2 and its translocation to the apical membrane. This results in reabsorption of water from the preurine [4]. In vascular endothelial cells, stimulation of V2R results in release of the contents of Weibel–Palade bodies (WPBs) into the circulation, causing a rise in VWF and FVIII plasma levels [5]. 1-Desamino-8-d-arginine vasopressin (desmopressin or DDAVP), a synthetic analog of AVP, is frequently used to treat patients with mild von Willebrand disease or hemophilia A [6]. Although it has been suggested that only 10% of VWF is secreted through storage organelles [7], other work has suggested that regulated secretion of VWF is important for plasma VWF levels [8]. A more recent study has shown that even spontaneously released VWF is, for

the large part, processed and secreted through post-Golgi storage organelles [9]. Altered V2R functioning could thus result in altered VWF and FVIII plasma levels.

We hypothesized that gain-of-function mutations in the X-chromosomal gene encoding V2R, *AVPR2*, will lead to increased VWF secretion, reflected in increased VWF propeptide levels, a measure of VWF secretion rate [10–12], resulting in increased levels of mature VWF and FVIII. To test this hypothesis, common single-nucleotide polymorphisms (SNPs) in *AVPR2* were genotyped in two different populations. Allelic distributions of these genotypes were used for association studies with VWF propeptide, VWF and FVIII plasma levels. *In vitro* studies were used to confirm SNP functionality.

Materials and methods

Study populations

Leiden Thrombophilia Study (LETS) The LETS consists of 474 consecutive patients with a first objectively confirmed episode of deep vein thrombosis and 474 healthy controls. For this study, we focused on controls only. The mean age of the control group was 45 years. The group consists of 272 women (57.4%) and 202 men (42.6%). Individuals with underlying malignancies were excluded. DNA samples are available for 471 controls. All participants were Caucasian and originated from The Netherlands. The design of this study has previously been described in detail [13,14]. FVIII antigen (FVIII:Ag), VWF antigen (VWF:Ag) and VWF propeptide were measured by enzyme-linked immunosorbent assay (ELISA) in the plasma of the first 301 controls included in the study, whereas FVIII activity (FVIII:C) was measured in the plasma of all controls by a one-stage clotting assay [15,16]. Pooled normal plasma, calibrated against the World Health Organization (WHO) standard for VWF and FVIII (91/666), was used as a reference. Results for FVIII:Ag, FVIII:C and VWF:Ag are expressed as international units per deciliter (U dL^{-1}). Results for VWF propeptide are expressed as units per deciliter (IU dL^{-1}), with 100 units being defined as the amount of VWF propeptide in 1 dL of pooled normal plasma.

Study of Myocardial Infarctions Leiden (SMILE) The SMILE consists of 560 patients, diagnosed with a first myocardial infarction, and 646 controls. For this study, we focused on controls only. The control group consists of men who were referred to the Leiden Anticoagulation Clinic for prophylactic anticoagulation therapy for a short period following minor orthopedic interventions. Controls had not used anticoagulants for a period of at least 6 months prior to participating in this study. The mean age of the control group was 57 years. All participants were Caucasian and originated from The Netherlands. Plasma and DNA samples were collected from all controls. Plasma FVIII:C was measured in two dilutions by a one-stage clotting assay with FVIII-deficient plasma and automated activated partial thromboplastin time (Organon Teknika, Boxtel, The Netherlands) on a STA (Diagnostic Stago; Boehringer Mannheim, Mannheim, Germany). VWF:Ag was measured by ELISA. Both FVIII:C and VWF:Ag levels are expressed as IU dL^{-1}. Pooled normal plasma, calibrated against an in-house standard that was calibrated against the WHO standard for VWF and FVIII (91/666), was used as a reference. Exclusion criteria for this study were renal disease, severe (neuro)psychiatric problems, or a life expectancy of < 1 year. The design of this study has previously been described in detail [17].

Both the LETS and the SMILE were approved by the appropriate ethical committees, and all participants gave informed consent.

Genetic studies

Genotyping On the basis of information on frequencies and prevalence of *AVPR2* SNPs in Caucasians available in dbSNP (www.ncbi.nlm.nih.gov/projects/SNP/) at the time, we initially selected the following *AVPR2* SNPs, located in coding regions only: S7T (ACT > TCT), G12E (GGG > GAG), L35L (CTG > CTA), V42A (GCG > GTG), G61V (GCC > GTC), V147A (GCG > GTG), L309L (CTA > CTG), and S331S (AGC > AGT). However, only G12E, L309L and S331S (rs2071126, rs5201 and rs5202, respectively) were prevalent in the LETS. Prevalent here means that the minor allele of an SNP was present in the LETS population. When a minor allele was not detected among the first 352 LETS samples tested, genotyping of that particular SNP was discontinued.

LETS controls were genotyped for these three SNPs using polymerase chain reaction (PCR) restriction fragment length polymorphism analysis. PCRs and enzymatic digestions were performed on a PTC-225 thermal cycler (Biozym, Hessisch Oldendorf, Germany), and primers were purchased from Eurogentec (Seraing, Belgium).

After resequencing of *AVPR2* as described below, the LETS population was genotyped for a fourth *AVPR2* variation, a-245c (rs4898372), by 5′-nuclease/Taqman assay. PCRs with fluorescent allele-specific oligonucleotide probes (Applied Biosystems, Foster City, CA, USA) were also performed on the PTC-225 thermal cycler. Fluorescence endpoint reading for allelic discrimination was performed on an ABI 7900 HT (Applied Biosystems).

Finally, we genotyped all four *AVPR2* SNPs in SMILE controls by 5′-nuclease/Taqman assay.

Sequencing The complete genomic region of *AVPR2* was resequenced in the DNA samples of the seven male LETS controls who were hemizygous for the rare allele of the *AVPR2* SNP S331S. A 3.3-kb region was amplified in fragments using five sets of primers. PCR products were purified using a QIAquick PCR Purification Kit (Qiagen Benelux, Venlo, The Netherlands). Sequence reactions and fragment analyses were performed by the Leiden Genome Technology Center (www.lgtc.nl, LGTC, Leiden, The Netherlands) on an ABI 3700 or ABI 3730 DNA Analyzer (Applied Biosystems).

In vitro studies

AVPR2 constructs An expression construct encoding wild-type V2R, C-terminally tagged with green fluorescent protein (GFP) (12G-V2R–GFP) [18], was kindly provided by A. Oksche (FMP, Berlin, Germany). The expression construct encoding 12E-V2R–GFP was made from 12G-V2R–GFP by site-directed mutagenesis, using a QuikChange II Site-Directed Mutagenesis Kit (Qiagen Benelux) and a set of 31-base mutagenesis primers (Eurogentec). Mutagenesis was confirmed by sequencing.

Cell culture Polarized Madin Darby Canine Kidney type II (MDCKII) cells, which, in contrast to Madin Darby Canine Kidney type I cells, lack endogenous V2R expression (kindly provided by A. Oksche), were cultured at 37 °C under 5% CO_2 in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, USA) supplemented with 5% fetal bovine serum, 1% non-essential amino acids, gentamicin, L-glutamine, and sodium bicarbonate. Cells were stably transfected with 12G-V2R–GFP or 12E-V2R–GFP constructs as described previously [19], and colonies were selected on the basis of V2R expression.

Immunoblotting and immunocytochemistry For immunoblotting, cells were lysed in Laemmli buffer containing 0.1 M dithiothreitol. High-mannose or total N-linked sugar moieties were removed with endoglycosidase H (Endo H) or protein *N*-glycosidase F (PNGase F) (both from New England Biolabs, Beverly, MA, USA), respectively, according to the manufacturer's protocol. Polyacrylamide gel electrophoresis, western blotting and immunodetection were performed as described elsewhere [19,20]. For detection of V2R–GFP, rabbit anti-GFP serum, diluted 1 : 5000, was used (B. Wieringa, Department of Cell Biology, RUNMC, Nijmegen, The Netherlands). As secondary antibodies, horseradish peroxidase-coupled goat anti-rabbit IgGs (Sigma, St Louis, MO, USA) were used. Immunocytochemistry, confocal laser-scanning microscopy and data quantification were performed as described elsewhere [18]. As primary antibody, rat anti-E-cadherin (Sigma), diluted 1 : 100, was used. As secondary antibody, goat anti-rat IgG coupled to Alexa 594, diluted 1 : 100, was used (Molecular Probes, Leiden, The Netherlands). Horizontal extended-focus images and vertical images were obtained with a Bio-Rad MRC-1000 laser-scanning confocal imaging system, using a × 60 oil-immersion objective, a 32 Kalman collection filter, an aperture diaphragm of 2.5, and an axial resolution of 0.14 μm per pixel. The images were contrast-stretched using Adobe Photoshop.

Radioligand-binding assay Untransfected MDCKII cells and 12G-V2R–GFP-expressing or 12E-V2R–GFP-expressing MDCKII cells were seeded at a density of 150 000 cells cm^{-2} on Costar filters (Corning, Corning, NY, USA) in 24-well plates, and grown to confluence over 3–4 days. Cells were washed twice in ice-cold phosphate-buffered saline with 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$ (PBS-CM), and plates were placed on ice, to prevent V2R internalization. Cells were incubated on the basolateral side with [^{3}H]AVP at concentrations ranging from 0 to 100 nM in PBS-CM for 2 h on ice. Cells were washed with PBS-CM, and filters were then excised and added to 5 mL of Filter-Count scintillation fluid (PerkinElmer, Waltham, MA, USA), and counted in a Tri-Card 1600 TR Liquid Scintillation Analyzer (PerkinElmer). Saturation curves of 12G-V2R–GFP and 12E-V2R–GFP were baseline corrected for values counted in untransfected cells to correct for non-specific binding. Three independent experiments were performed in duplicate.

cAMP assay Untransfected MDCKII cells and 12G-V2R–GFP-expressing or 12E-V2R–GFP-expressing MDCKII cells were seeded at a density of 600 000 cells cm^{-2} on Costar filters (Corning) in 24-well plates and grown to confluency overnight in the presence of [^{3}H]adenine (2 μCi mL^{-1}). Cells were washed twice with HBS buffer [25 mM HEPES, pH 7.2, supplemented with 0.75 mM NaH_2PO_4, 140 mM NaCl, and 0.05% (w/v) bovine serum albumin] and preincubated at 37 °C with 1 mM 3-isobutyl-1-methylxanthine in HBS buffer. After 30 min, 25 μL of concentrated AVP solution was added basolaterally, resulting in final AVP concentrations, on the basolateral side of the filter, ranging from 0.0001 to 100 nM. Cells were incubated for an additional 25 min at 37 °C. Then, cells were placed on ice, and the HBS buffer was removed. Cells were lysed in 1 mL (0.25 mL apically and 0.75 mL basolaterally) of 5% (w/v) trichloroacetic acid, supplemented with 0.1 mM cAMP and 0.1 mM ATP, for at least 30 min. Lysates were stored at − 20 °C, and cAMP was measured within 1 week.

To measure [^{3}H]cAMP generation, lysates were loaded onto Dowex 50W-X4 columns (Bio-Rad, Hercules, CA, USA), followed by 2 mL of water, with the flow-through being discarded. Then, the Dowex columns were placed over alumina columns (Sigma) and eluted with 10 mL of water, with the flow-through again being discarded. The alumina columns were then placed over scintillation vials and eluted with 6 mL of 0.1 M imidazole (Sigma). Fifteen milliliters of scintillation fluid (Ecoscint XR; National Diagnostics, Atlanta, GA, USA) was added to each vial, and tritium was counted for 5 min on a Wallac 1409 Liquid Scintillation Counter. Dowex columns were regenerated by adding 10 mL of 2 M HCl followed by 10 mL of water; the alumina columns were regenerated by adding 2 mL of 1 M imidazole, 10 mL of 0.1 M imidazole, and finally 5 mL of water. Three independent experiments were performed in duplicate.

Statistical analysis

To evaluate differences in VWF propeptide, VWF and FVIII levels between genotypes, Student's *t*-tests and linear regression modeling were used. Differences in mean levels, together with 95% confidence intervals (CIs) of these differences, are given. Differences in mean levels between groups were adjusted for

age and ABO blood group by linear regression modeling. Because *AVPR2* is located on the X-chromosome, analyses were stratified on sex.

Data for AVP saturation experiments in MDCKII cells were analyzed with GRAPHPAD PRISM. Maximum binding capacity (B_{max}) and dissociation constant (K_D) were determined, along with corresponding 95% CIs. The difference between K_D values was evaluated with a paired, two-tailed *t*-test. For cAMP, $-\log EC_{50}$ values were calculated with their corresponding standard deviations, using GRAPHPAD PRISM. We used a paired, one-tailed *t*-test to evaluate whether cAMP generation was increased in 12E-V2R–GFP cells as compared with 12G-V2R–GFP cells.

Results

Genotyping

Initially, we genotyped the LETS controls for three *AVPR2* SNPs: G12E, L309L, and S331S. As described below, G12E and S331S were associated with the levels of VWF propeptide, VWF, and FVIII. We resequenced the entire *AVPR2* genomic region in all men who were hemizygous for these two SNPs in the LETS, to identify variations linked to G12E and S331S. We identified one other potentially functional polymorphism, a-245c. Therefore, we additionally genotyped LETS and SMILE controls for this SNP.

Genotyping was successful in all LETS controls, except for a-245c in one individual. In the female controls, all SNPs were in Hardy–Weinberg equilibrium. Minor allele frequencies (MAFs) of three of the SNPs, a-245c, G12E, and S331S, were low, namely 2.43%, 1.89%, and 2.30%, respectively, in healthy controls. L309L was more common, and had a MAF of 25.95% in healthy controls. In the SMILE, correct genotypes are missing in 36 controls for a-245c, in 27 for G12E, in 21 for L309L, and in 15 for S331S, owing to sample depletion. MAFs in controls were slightly lower than in the LETS, namely, approximately 1.15%, 0.97% and 1.11% for a-245c, G12E, and S331S, respectively, and 27.20% for L309L.

SNPs a-245c, G12E and S331S were strongly linked to each other. Furthermore, rare alleles of these SNPs only occurred in individuals also carrying rare alleles of L309L. In Table 1, we show the *AVPR2* haplotypes identified in the LETS and the SMILE.

Table 1 *AVPR2* haplotypes identified in the Leiden Thrombophilia Study and the Study of Myocardial Infarctions Leiden

Haplotype*	*AVPR2* a-245c	*AVPR2* G12E	*AVPR2* L309L	*AVPR2* S331S
1	A	GGG	CTA	AGC
2	A	GGG	CT**G**	AGC
3	**C**	GGG	CT**G**	AGC
4	A	GGG	CT**G**	AG**T**
5	**C**	GGG	CT**G**	AG**T**
6	**C**	GAG	CT**G**	AG**T**

*Haplotypes fit all but one individual; bold print indicates a nucleotide change.

Associations between AVPR2 SNPs and levels of VWF propeptide, VWF, and FVIII

Healthy LETS controls In men, associations with levels of VWF propeptide were observed for three SNPs: a-245c, G12E, and S331S (Table 2A). Similar associations with VWF and FVIII were indicated. Men hemizygous for the rare alleles of these SNPs had higher levels of VWF propeptide, VWF and FVIII:Ag than men hemizygous for the common alleles. FVIII:C appeared to be elevated as well, but to a lesser extent. L309L was not associated with VWF propeptide, VWF and FVIII levels in male controls. Associations between a-245c, G12E and S331S and levels were not present in women (Table 2B). However, slight increases in VWF and FVIII levels observed in women heterozygous for these three SNPs could suggest a gene dosage effect. Among female LETS controls, there were no homozygous carriers of the rare alleles of a-245c, G12E, or S331S. Adjustment for age and ABO blood group affected outcomes in neither men nor women. In a linear regression model, it is predicted that this cluster of SNPs (a-245c, G12E, and S331S) can explain 2.5% of total variation in VWF propeptide in the LETS, 1.8% of the variation in VWF:Ag, and 2.7% of the variation in FVIII:Ag.

Healthy SMILE controls The SMILE control population was analyzed to replicate the observations in the LETS. Although the SMILE includes only men and lacks measurements of VWF propetide and FVIII:Ag, we confirmed that control subjects hemizygous for the rare alleles of a-245c, G12E and S331S had higher levels of VWF than those carrying the common alleles (Table 2C). The effects on FVIII:C levels were much more moderate. In a linear regression model, it is predicted that this cluster of SNPs explains 3.2% of the variation in VWF:Ag and 1.3% of the variation in FVIII:C in the SMILE. In contrast to the LETS, an association with increased VWF and FVIII levels was also observed for the rare allele of L309L, although the effect was smaller than for the other SNPs. Adjustment for neither age nor ABO blood group affected the outcomes.

In vitro *studies*

Transfection of MDCKII cells Of the three SNPs that are associated with VWF propeptide, VWF and FVIII plasma levels, G12E was the only coding, non-synonymous SNP. Therefore, it was the most likely candidate for an effect on V2R functioning. The potential functionality of G12E was investigated in an *in vitro* model. MDCKII cells were transfected with either 12G-V2R–GFP or 12E-V2R–GFP, and stable cell lines were formed. Immunocytochemical findings showed that both 12G-V2R–GFP and 12E-V2R–GFP colocalized with the marker protein E-cadherin on the basolateral membrane of the cells, whereas untransfected

Table 2A Mean levels of von Willebrand factor (VWF) propeptide, VWF and FVIII in healthy male controls; the Leiden Thrombophilia Study

		VWF propeptide (U dL^{-1})			VWF:Ag (IU dL^{-1})			FVIII:Ag (IU dL^{-1})			FVIII:C (IU dL^{-1})			
Genotype*	*N*	Mean	Δ	95% CI	Mean	Δ	95% CI	Mean	Δ	CI95	*N*	Mean	Δ	95% CI
a-245c														
A†	115	114	–	–	127	–	–	114	–	–	193	119	–	–
C	5	145	31	8–53	154	28	− 8–63	147	33	− 3–69	6	141	22	− 5–48
G12E														
G†	116	114	–	–	127	–	–	114	–	–	195	120	–	–
A	4	139	25	1–50	146	18	− 21–58	140	26	− 15–66	5	129	9	− 20–38
L309L														
A†	97	115	–	–	129	–	–	116	–	–	153	120	–	–
G	23	115	0	− 11–12	121	− 9	− 27–9	111	− 5	− 24–13	47	118	− 2	− 13–8
S331S														
C†	115	114	–	–	127	–	–	114	–	–	193	119	–	–
T	5	145	31	8–53	154	28	− 8–63	147	33	− 3–69	7	135	16	− 9–41

CI, confidence interval; FVIII:Ag, FVIII antigen; FVIII:C, FVIII activity; VWF:Ag, VWF antigen. *As the *AVPR2* gene is located on the X-chromosome, only single alleles are shown for male study participants. †Reference group. Δ represents the mean difference from the reference group.

Table 2B Mean levels of von Willebrand factor (VWF) propeptide, VWF and FVIII in healthy female controls; the Leiden Thrombophilia Study

		VWF propeptide (U dL^{-1})			VWF:Ag (IU dL^{-1})			FVIII:Ag (IU dL^{-1})			FVIII:C (IU dL^{-1})			
Genotype	*N*	Mean	Δ	95% CI	Mean	Δ	95% CI	Mean	Δ	95% CI	*N*	Mean	Δ	95% CI
a-245c														
AA*	171	108	–	–	117	–	–	103	–	–	258	123	–	–
AC	8	101	− 8	− 25–9	119	3	− 24–29	109	6	− 22–34	12	130	7	− 12–26
G12E														
GG*	173	108	–	–	117	–	–	103	–	–	261	123	–	–
GA	6	100	− 8	− 28–12	127	10	− 21 to − 40	123	19	− 13–52	9	138	15	− 14–44
L309L														
AA*	99	110	–	–	120	–	–	108	–	–	146	124	–	–
AG	69	106	− 5	− 12–3	110	− 10	− 21 to − 2	95	− 13	− 25 to − 1	103	119	− 5	−13–3
GG	11	104	− 6	− 11–12	140	20	− 4–45	117	9	− 16–35	21	139	15	− 1–30
S331S														
CC*	173	109	–	–	117	–	–	103	–	–	260	123	–	–
CT	6	100	− 8	− 16–0	127	10	− 21–40	123	19	− 13–52	10	136	13	− 8–34

CI, confidence interval; FVIII:Ag, FVIII antigen; FVIII:C, FVIII activity; VWF:Ag, VWF antigen. *Reference group. Δ represents the mean difference from the reference group.

control cells revealed no GFP signal (Fig. 1). In both transfected cell lines, late endosomal/lysosomal localization of V2R–GFP was observed, indicating that receptor internalization occurred for both V2R variants (data not shown). For AVP binding and cAMP generation experiments (described below), GFP-positive colonies of both 12G-V2R–GFP and 12E-V2R–GFP expressing cells with similar V2R–GFP expression levels were selected visually.

To test whether glycosylation was normal, we treated total cell lysates with Endo H and PNGase F to cleave off N-linked high-mannose sugar groups (indicative of V2R localized to the endoplasmic reticulum) or all N-linked sugar moieties, respectively. GFP immunoblotting of cell lysates revealed that the extent and form of glycosylation were similar to that of wild-type V2R, as shown previously [20], indicating that the processing of 12E-V2R is similar to that of 12G-V2R.

V2R binding affinity V2R saturation experiments with [$_3$H]AVP show similar B_{max} values for both 12G-V2R and 12E-V2R cell lines, which were reached at an AVP concentration of approximately 50–60 nM (Fig. 2A). This indicates similar V2R expression levels in both cell lines. However, the saturation curve of 12E-V2R is steeper than that of 12G-V2R. The K_D was 4.5 nM (95% CI 3.6–5.4) for 12E-V2R and 16.5 nM (95% CI 10.1–22.9) for 12G-V2R, corresponding to a three-fold increase in AVP binding affinity for 12E-V2R as compared with 12G-V2R ($P < 0.0001$).

cAMP generation To test whether increased AVP binding affinity also leads to increased intracellular signal transduction, we measured intracellular cAMP generation at increasing concentrations of AVP (Fig. 2B). Both receptors were

Table 2C Mean levels of von Willebrand factor (VWF) and FVIII (IU mL^{-1}) in healthy male controls; the Study of Myocardial Infarctions Leiden

	VWF:Ag (IU dL^{-1})				FVIII:C (IU dL^{-1})			
Genotype*	*N*	Mean	Δ	95% CI	*N*	Mean	Δ	95% CI
a-245c								
A†	576	131	–	–	602	123	–	–
C	7	220	89	41–136	7	125	3	– 22–27
G12E								
G†	586	131	–	–	612	123	–	–
A	6	242	110	60–161	6	130	7	– 19–33
L309L								
A†	434	128	–	–	455	122	–	–
G	163	144	16	4–27	169	125	2	– 3–8
S331S								
C†	596	131	–	–	623	123	–	–
T	7	220	89	42–136	7	125	2	– 23–27

CI, confidence interval; FVIII:Ag, FVIII antigen; FVIII:C, FVIII activity; VWF:Ag, VWF antigen. *As the *AVPR2* gene is located on the X-chromosome, only single alleles are shown for male study participants. †Reference group. Δ represents the mean difference from the reference group.

functional, and they gave a similar maximal response for cAMP generation at 100 nM AVP. The 12E-V2R–GFP cells were more potent in their cAMP response at lower concentrations of AVP. The $-\log EC_{50}$ was 10.38 ± 1.35 for 12E-V2R–GFP, compared with 8.72 ± 0.56 for wild-type 12G-V2R–GFP ($P = 0.05$). This shows that the increased binding affinity of 12E-V2R for AVP does indeed result in increased intracellular signal transduction in MDCKII cells. Untransfected MDCKII cells showed no cAMP generation in response to AVP, even at 100 nM.

Discussion

We hypothesized that gain-of-function variations in the *AVPR2* gene would lead to increased VWF secretion from WPBs and to increased plasma levels of VWF and FVIII. We observed that the rare alleles of three *AVPR2* SNPs, a-245c, G12E, and S331S, were associated with increased levels of VWF propeptide in the LETS and increased mature VWF levels in the SMILE. The three SNPs were in strong linkage disequilibrium, but none of the *AVPR2* SNPs was linked to three SNPs in the adjacent *F8* gene [D1241E (rs1800291), G24052A (rs6655259) and G27882C (rs28370236)], which have previously been shown to associate with FVIII levels in the LETS and the SMILE [21,22].

Because these three *AVPR2* SNPs were in strong linkage disequilibrium, it is likely that the observed associations were caused by only one SNP. G12E was the most likely candidate for functional variation, as G12E is a coding non-synonymous SNP, located in the N-terminal extracellular tail, that may influence ligand binding. We demonstrated a three-fold increase in the binding affinity of 12E-V2R for AVP as compared with wild-type 12G-V2R in stably transfected MDCKII cells, and a corresponding increase in intracellular cAMP generation. This means that, even though both alleles of the SNP yield a fully functional receptor, carriers of the E-allele will be able to respond to lower plasma concentrations of AVP.

The associations that we observed with plasma levels were strongest for VWF propeptide in the LETS and for mature VWF in the SMILE in men. Associations with FVIII:Ag and FVIII:C were indicated. This suggests that VWF secretion is indeed upregulated. Although there were no women homozygous for G12E in the LETS, there did appear to be an intermediate effect on VWF and FVIII levels in heterozygous women.

It should be noted, however, that the numbers of G12E carriers are small – only five hemizygous male and nine heterozygous female controls in the LETS, and six hemizygous male controls in the SMILE. However, CIs around the differences in VWF propeptide levels in men in the LETS and VWF levels in the SMILE were narrow. Furthermore, the fact that similar associations were observed in two independent study populations supports the validity of these observations. Although the effects on VWF levels in male controls were larger in the SMILE than in the LETS, this discrepancy can, in

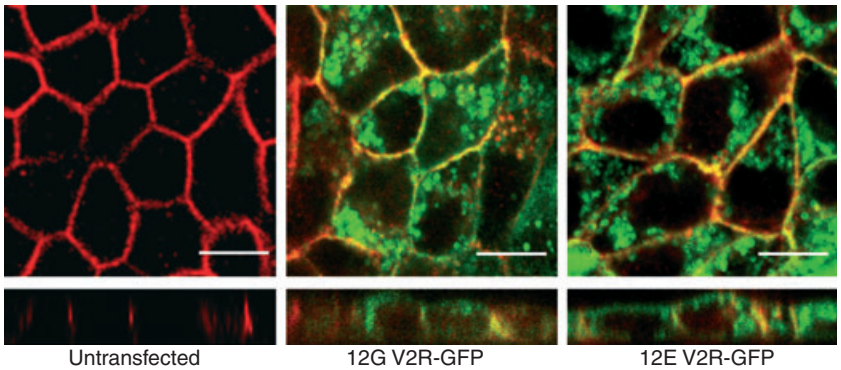

Fig. 1. Arginine vasopressin 2 receptor (V2R)–green fluorescent protein (GFP) expressed in Madin Darby Canine Kidney type II (MDCKII) cells. Localization of 12G-V2R–GFP and 12E-V2R–GFP (green) and the basolateral plasma membrane marker E-cadherin (red) in untransfected and stably transfected MDCKII cells. Cells were visualized from above and from the side, and colocalization is visble in yellow. As primary antibody, rat anti-E-cadherin, diluted 1 : 100, was used. As secondary antibody, goat anti-rat IgG coupled to Alexa 594, diluted 1 : 100, was used. The images were contrast-stretched using Adobe Photoshop. The white size bars correspond to a distance of 10 μm.

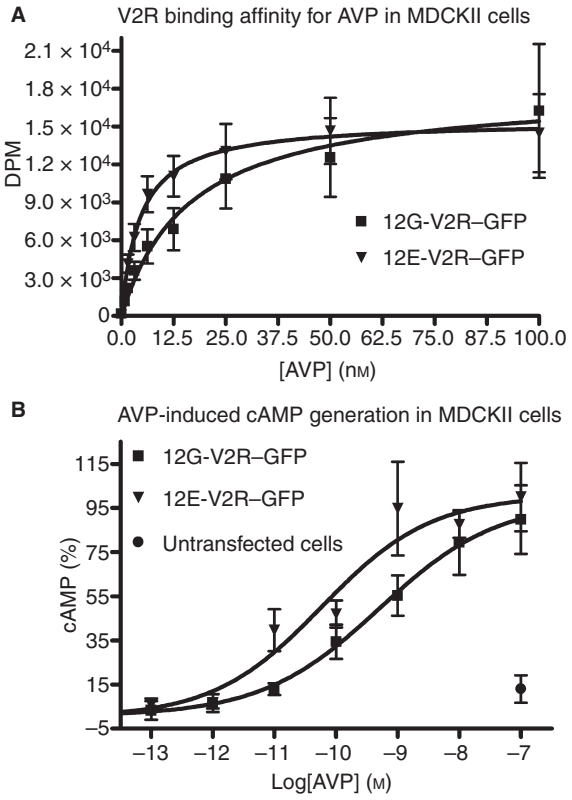

Fig. 2. Arginine vasopressin 2 receptor (V2R) binding affinity for arginine vasopressin (AVP) and AVP-induced cAMP generation in Madin Darby Canine Kidney type II (MDCKII) cells. (A) Saturation curves of 12G-V2R–green fluorescent protein (GFP) and 12E-V2R–GFP with [^{3}H]AVP in stably transfected MDCKII cells. Untransfected MDCKII cells and cells transfected with 12G-V2R–GFP or 12E-V2R–GFP, grown to confluence on Costar filters, were treated with [^{3}H]AVP on the basolateral side at concentrations ranging from 0 to 100 nM in phosphate-buffered saline with 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$ for 2 h on ice. Filters were washed and excised, and tritium was counted in Filter-Count scintillation fluid. Radioactivity is expressed as disintegrations per minute (DPM). Three independent experiments were performed in duplicate. Both curves are baseline corrected for values counted in untransfected cells to correct for non-specific binding. (B) cAMP generation in untransfected, 12G-V2R–GFP-expressing and 12E-V2R–GFP-expressing MDCKII cells upon stimulation with increasing concentrations of AVP. Untransfected, 12G-V2R–GFP-expressing or 12E-V2R–GFP-expressing MDCKII cells, grown to confluency on filters in the presence of [^{3}H]adenine, were stimulated with AVP at concentrations ranging from 0.0001 to 100 nM, after preincubation with 3-isobutyl-1-methylxanthine. [^{3}H]cAMP was purified from cell lysates and quantified by counting tritium. Three independent experiments were performed in duplicate. cAMP is expressed in percentages, where 0% represents the lowest number of counts and 100% the highest number of counts measured. AVP is expressed in exponents of the molar concentration.

part, be explained by a difference in age between male controls hemizygous for G12E (59 years in the SMILE vs. 51 years in the LETS). Finally, the *in vitro* data for G12E support the associations with levels observed in both studies.

We conclude that *AVPR2* variations are modifiers of plasma levels of VWF propeptide and VWF. The *AVPR2* G12E variant is a gain-of-function mutation that leads to normally expressed, fully functional V2R with increased binding affinity for AVP, and consequently with increased signal transduction as reflected by higher intracellular cAMP generation. As 12E-V2R can readily be activated by low levels of AVP, this variation may lead to increased VWF secretion from WPBs, which explains the association of G12E with higher VWF propeptide and VWF plasma levels.

Addendum

A. Y. Nossent designed the study, collected and analyzed data, and wrote the manuscript; J. H. Robben collected and analyzed data and edited the manuscript; P. M. T. Deen designed the study and edited the manuscript; H. L. Vos designed the study and edited the manuscript; F. R. Rosendaal designed the study and edited the manuscript; C. J. M. Doggen designed the study, collected data, and edited the manuscript; J. L. Hansen designed the study, analyzed data, and edited the manuscript; S. P. Sheikh designed the study and edited the manuscript; R. M. Bertina designed the study and edited the manuscript; H. C. J. Eikenboom designed the study, analyzed data, and edited the manuscript.

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Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

References

1 Kamphuisen PW, Houwing-Duistermaat JJ, van Houwelingen HC, Eikenboom HC, Bertina RM, Rosendaal FR. Familial clustering of factor VIII and von Willebrand factor levels. *Thromb Haemost* 1998; **79**: 323–7.
2 Kamphuisen PW, Lensen R, Houwing-Duistermaat JJ, Eikenboom JC, Harvey M, Bertina RM, Rosendaal FR. Heritability of elevated factor VIII antigen levels in factor V Leiden families with thrombophilia. *Br J Haematol* 2000; **109**: 519–22.
3 De Visser MC, Sandkuijl LA, Lensen RP, Vos HL, Rosendaal FR, Bertina RM. Linkage analysis of factor VIII and von Willebrand factor loci as quantitative trait loci. *J Thromb Haemost* 2003; **1**: 1771–6.
4 Knoers NV, Deen PM. Molecular and cellular defects in nephrogenic diabetes insipidus. *Pediatr Nephrol* 2001; **16**: 1146–52.
5 Kaufmann JE, Oksche A, Wollheim CB, Gunther G, Rosenthal W, Vischer UM. Vasopressin-induced von Willebrand factor secretion from endothelial cells involves V2 receptors and cAMP. *J Clin Invest* 2000; **106**: 107–16.
6 Mannucci PM. Desmopressin (DDAVP) for treatment of disorders of hemostasis. *Prog Hemost Thromb* 1986; **8**: 19–45.
7 Sporn LA, Marder VJ, Wagner DD. Inducible secretion of large, biologically potent von Willebrand factor multimers. *Cell* 1986; **46**: 185–90.

8 Tsai HM, Nagel RL, Hatcher VB, Seaton AC, Sussman II. The high molecular weight form of endothelial cell von Willebrand factor is released by the regulated pathway. *Br J Haematol* 1991; **79**: 239–45.
9 Giblin JP, Hewlett LJ, Hannah MJ. Basal secretion of von Willebrand factor from human endothelial cells. *Blood* 2008; **112**: 957–64.
10 Borchiellini A, Fijnvandraat K, ten Cate JW, Pajkrt D, van Deventer SJ, Pasterkamp G, Meijer-Huizinga F, Zwart-Huinink L, Voorberg J, van Mourik JA. Quantitative analysis of von Willebrand factor propeptide release in vivo: effect of experimental endotoxemia and administration of 1-deamino-8-D-arginine vasopressin in humans. *Blood* 1996; **88**: 2951–8.
11 van Mourik JA, Boertjes R, Huisveld IA, Fijnvandraat K, Pajkrt D, van Genderen PJ, Fijnheer R. von Willebrand factor propeptide in vascular disorders: a tool to distinguish between acute and chronic endothelial cell perturbation. *Blood* 1999; **94**: 179–85.
12 Vischer UM, Ingerslev J, Wollheim CB, Mestries JC, Tsakiris DA, Haefeli WE, Kruithof EK. Acute von Willebrand factor secretion from the endothelium in vivo: assessment through plasma propeptide (vWf:AgII) levels. *Thromb Haemost* 1997; **77**: 387–93.
13 Koster T, Rosendaal FR, de Ronde H, Briët E, Vandenbroucke JP, Bertina RM. Venous thrombosis due to poor anticoagulant response to activated protein C: Leiden Thrombophilia Study. *Lancet* 1993; **342**: 1503–6.
14 van der Meer FJ, Koster T, Vandenbroucke JP, Briët E, Rosendaal FR. The Leiden Thrombophilia Study (LETS). *Thromb Haemost* 1997; **78**: 631–5.
15 Koster T, Blann AD, Briët E, Vandenbroucke JP, Rosendaal FR. Role of clotting factor VIII in effect of von Willebrand factor on occurrence of deep-vein thrombosis. *Lancet* 1995; **345**: 152–5.
16 Nossent AY, van Marion V, van Tilburg NH, Rosendaal FR, Bertina RM, van Mourik JA, Eikenboom HC. von Willebrand factor and its propeptide: the influence of secretion and clearance on protein levels and the risk of venous thrombosis. *J Thromb Haemost* 2006; **4**: 2556–62.
17 Doggen CJ, Kunz G, Rosendaal FR, Lane DA, Vos HL, Stubbs PJ, Manger Cats V, Ireland H. A mutation in the thrombomodulin gene, 127G to A coding for Ala25Thr, and the risk of myocardial infarction in men. *Thromb Haemost* 1998; **80**: 743–8.
18 Robben JH, Knoers NV, Deen PM. Characterization of vasopressin V2 receptor mutants in nephrogenic diabetes insipidus in a polarized cell model. *Am J Physiol Renal Physiol* 2005; **289**: F265–72.
19 Deen PM, Van Balkom BW, Savelkoul PJ, Kamsteeg EJ, Van Raak M, Jennings ML, Muth TR, Rajendran V, Caplan MJ. Aquaporin-2: COOH terminus is necessary but not sufficient for routing to the apical membrane. *Am J Physiol Renal Physiol* 2002; **282**: F330–40.
20 Robben JH, Knoers NV, Deen PM. Regulation of the vasopressin V2 receptor by vasopressin in polarized renal collecting duct cells. *Mol Biol Cell* 2004; **15**: 5693–9.
21 Nossent AY, Eikenboom HC, Vos HL, Bakker E, Tanis BC, Doggen CJ, Bertina RM, Rosendaal FR. Haplotypes encoding the factor VIII 1241 Glu variation, factor VIII levels and the risk of venous thrombosis. *Thromb Haemost* 2006; **95**: 942–8.
22 Nossent AY, Eikenboom HC, Tanis BC, Doggen CJ, Rosendaal FR. Haplotypes encoding the factor VIII 1241Glu variation and the risk of myocardial infarction. *J Thromb Haemost* 2007; **5**: 619–21.