Novel insights in thrombosis pathophysiology using Mice with Impaired anticoagulation

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Circulating Nucleosomes and Elastase α1-Antitrypsin Complexes and the Novel Thrombosis Susceptibility Locus *SLC44A2*

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Venous Thromboembolism (VTE) is the third most common cardiovascular cause of death, and genetic and environmental risk factors are involved in its pathophysiology (1, 2). Thus far, all established genetic VTE risk factors appeared to be directly related to blood coagulation (3). However, a recent meta-analysis of twelve genome wide association studies (GWASs), in which 7,507 VTE-affected individuals and 52,632 control subjects were included, discovered novel loci associated with VTE that cannot be related (yet) to the hemostatic system (4). One of these loci harbors the Solute carrier 44a2 (*SLC44A2*) gene, which encodes a choline transporter. The exonic SNP with the highest risk identified on *SLC44A2* was rs2288904, with the major allele (adenine (A) over guanine (G)) increasing the risk of VTE. Within the GWAS meta-analysis the odds ratio (OR) of this SNP for disease was 1.21 (P=2.75 $\times 10^{-15}$), and its association was confirmed in three separate replication studies (combined: 3,009 VTE-affected individuals and 2,586 control subjects). Interestingly, the association between *SLC44A2* and thrombosis was recently confirmed in a separate study (5).

The non-synonymous lead SNP rs2288904 at the *SLC44A2* locus has been causally related with transfusion-related acute lung injury (TRALI) (6). *SLC44A2*/rs2288904 (A or G) produces an amino acid substitution in the extracellular domain of the *SLC44A2* protein (Arg154Gln). This substitution can trigger (allo-)antibody formation in carriers of the minor (A) allele (during pregnancy and exposure to the major (G) allele variant). Subsequently, upon plasma transfusion these antibodies can trigger TRALI. Although the exact sequence of events is not entirely clear, the relation between rs2288904 and TRALI is well-established. Several clinical studies demonstrated that neutrophils play a key role in TRALI formation (7-9). Moreover, in experimental studies it has been shown that during TRALI neutrophils are activated and neutrophil extracellular traps (NETs) are formed, which mediate the inflammatory response. These TRALI symptoms can be treated with specific agents targeting NET components (10, 11).

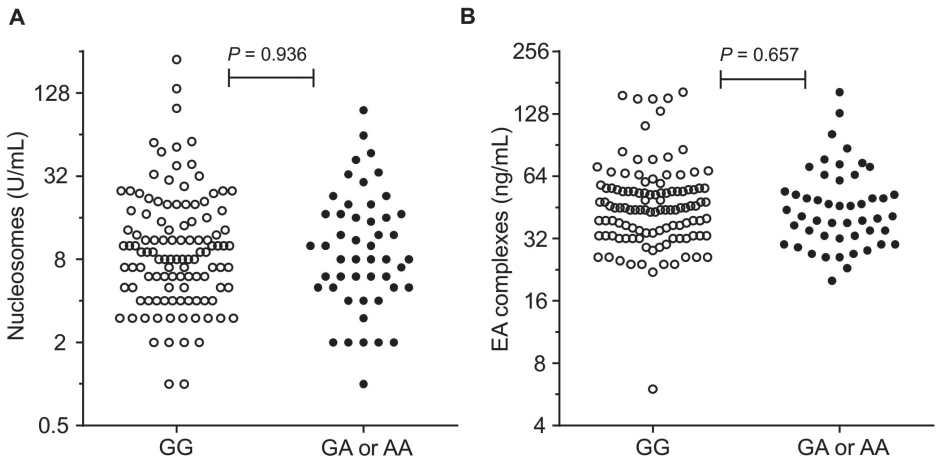
Interestingly, neutrophil activation and NET formation are also linked to VTE development in mouse models, with NET inhibition reducing thrombus formation (12). Moreover, there are claims NET markers are elevated in human VTE patients (13, 14). In the present study, the relation between neutrophil activation and NET formation in VTE and *SLC44A2*/rs2288904 is investigated. Systemic neutrophil activation was evidenced by the presence of circulating elastase α1-antitrypsin (EA) complexes (13). Nucleosome levels in plasma have been reported to be a suitable marker for NET formation in plasma in humans (15).

Because of the association of *SLC44A2*/rs2288904 with VTE and TRALI, and considering the involvement of NETs in both diseases, we hypothesized that *SLC44A2*/rs2288904 genotype modifies neutrophil activation and NET formation. Reduced neutrophil activation and NET formation might consequently be the cause of a protective effect of rs2288904-A (the minor allele) in

its association with VTE. To test this hypothesis, individuals from a previously characterized VTE study population, in whom levels of circulating nucleosomes and EA complexes have been determined, were genotyped for rs2288904 (13). In this cohort it was demonstrated that circulating nucleosomes and EA complexes were increased in deep vein thrombosis (DVT) patients compared to individuals with a suspicion of DVT in whom the diagnosis was ruled out (13). Of note, nucleosomes and EA complexes were measured in plasma obtained from blood without any additional (neutrophil) stimulants. Because *SLC44A2*/rs2288904-A was found to be protective for VTE, we assumed that either one or two copies of this allele are required to decrease the VTE risk. Therefore, we tested for a dominant effect of allele A on the plasma levels of nucleosomes and EA complexes.

We successfully genotyped 162 control subjects and 128 VTE patients from a total of 307 available DNA samples. Genotyping was performed using the Taqman SNP genotyping Assay (Life Technologies, Carlsbad (CA), USA), according to the manufacturers protocol. In the control population (no VTE upon examination), median nucleosomes and EA complex levels of the GG population were 9 U/mL (1-244 U/mL) and 45 ng/mL (6-163 ng/mL), respectively (figure 1A and B). In the combined GA and AA population, levels of circulating nucleosomes and EA complexes were not significantly increased (median nucleosomes: 8 U/mL (1-96 U/mL), P=0.936, and median EA complexes 41 ng/mL (20-163 ng/mL), P=0.657). Moreover, within the VTE patient population or in the two populations combined (290 individuals) no differences were found between the two genotypes (figure 1C: P=0.716 and P=0.413, nucleosomes and EA complexes, within VTE patients, respectively. P=0.575 and P=0.714, nucleosomes and EA complexes, respectively, within all individuals). There were no differences found in circulating nucleosomes and EA complexes between GA and AA individuals in all three groups i.e. the control population, the VTE patient population, and the two populations combined (see legends of figure 1). In conclusion, these results indicate that nucleosome and EA complex levels are not depending on *SLC44A2*/rs2288904 genotype.

Germain et al. demonstrated an overrepresentation of rs2288904 G over A in VTE individuals, both in the meta-analysis (OR: 1.19, (Confidence interval (CI): 1.12-1.26, α=0.05), P=1.07 x10^{-9}) as well as in replication studies (OR: 1.28, (CI: 1.16-1.40, α=0.05), P=2.64 x10^{-7}), illustrating the robustness of the observation (4). However, for the study group used here we were unable to reproduce this observation (OR: 0.86, (CI: 0.47-1.56, α=0.05), P=0.623). Whether this is due to the small samples size in the present study (5,595 vs. 307 individuals, in the replication study and our study population, respectively) or differences between the study populations (healthy controls vs. controls suspected of VTE, but in whom the diagnosis was ruled out, in the replication study and our study population, respectively) is subject to speculation. However, despite the lack of



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	Nucleosomes (U/mL)			EA complexes (ng/mL)		
Population (n)	GG	GA or AA	*P*-value	GG	GA or AA	*P*-value
Control (162)	9 (1-244)	8 (1-96)	0.936	45.0 (6-163)	41.0 (20-163)	0.657
VTE patients (128)	17 (2-323)	17 (1-190)	0.716	51.0 (2-220)	57.0 (25-259)	0.413
Total (290)	11 (1-323)	11.5 (1-190)	0.575	48.0 (2-220)	47.5 (20-259)	0.714

Figure 1 | Nucleosome and elastase α1-antitrypsin complex levels in plasma are not altered in individuals with a different rs2288904 allele. A) Plasma nucleosome levels in either GG carrying individuals (open circles, 113 individuals) or GA or AA carrying individuals (filled dots, 49 individuals). G: guanine, A: adenine. Nucleosome levels were measures as described (13). B) Plasma elastase α1-antitrypsin (EA) complex levels in plasma in either GG carrying individuals (major allele, open circles, 113 individuals) or GA or AA carrying individuals (minor allele, filled dots, 49 individuals). EA complex levels were measures as described (13). C) Overview of nucleosome and EA complex levels in plasma in the control population, the VTE patient population, and the total population (the previous two populations combined). No significant differences were found between GA and AA individuals. Control group (nucleosomes and EA complexes, respectively): 9 U/mL (1-96 U/mL) and 8 U\mL (5-63 U/mL), and 42.5 ng/mL (20-163 ng/mL) and 38 ng/mL (33-71 ng/mL), *P*=0.917). VTE patient population: 16.5 U/mL (1-190 U/mL) and 21 U\mL (3-57 U/mL), and 56 ng/mL (25-259 ng/mL) and 70 ng/mL (37-156 ng/mL). Two populations combined: 11 U/mL (1-190 U/mL) and 16.5 U\mL (3-63 U/mL), and 47.5 ng/mL (20-259 ng/mL) and 50 ng/mL (33-156 ng/mL). Number of individuals per group are as follows: Control; 162 individuals in total, 113 GG and 49 GA or AA (allele frequency G (A): 82.7% (17.3 %)). VTE patients; 128 individuals in total, 85 GG and 43 GA or AA (allele frequency G (A): 80.5% (19.5%)). Total; 290 individuals in total, 198 GG and 92 GA or AA (allele frequency G (A): 81.7% (18.3%)). For statistical analyses a Mann Whitney Rank-sum test was used. Data are presented as the median with the range (minimum and maximum, respectively).

association of *SLC44A2*/rs2288904 with VTE in the present study, our observation remains that nucleosome and EA complex levels are not influenced by rs2288904 genotype.

The range of nucleosomes and EA complexes is large within this specific cohort, both in controls (individuals with a suspicion of DVT in whom the diagnosis was ruled out) and in cases. This was previously attributed to comorbidities and other health conditions (e.g. malignancy or recent surgery), which were present in both groups (13). Here, we considered that variation in *SLC44A2* (on rs2288904) partly explains the observed the variation nucleosomes and EA complexes, however, that was not true for the present cohort.

To avoid selection bias, conclusions regarding the association of circulating nucleosomes and EA complexes and rs2288904 should only be drawn from observations in the control study population only. However, also for the total and the VTE patient population only, no effect of rs2288904 genotype on plasma levels of nucleosomes and EA complexes was found.

Although circulating nucleosomes and EA complexes are at present the best characterized biomarkers for NET formation and neutrophil activation, their lack of association with rs2288904 genotype does not exclude a role for *SLC44A2* in neutrophil activation (and possibly NET formation) in VTE. Studying ex vivo activation of isolated neutrophils from carriers of each genotype is an alternative strategy to study the link between *SLC44A2*/rs2288904, VTE, and neutrophil activation. Such studies can include impact of different genotypes on NET formation, interaction of neutrophils with endothelium, and other functional aspects of neutrophils. Alternative hypotheses explaining the association of *SLC44A2*/rs2288904 with VTE, apart from neutrophil activation, may involve von Willebrand Factor (VWF) or its choline transporter function. It has recently been described SLC44A2 directly interacts with VWF (16), a protein essential for hemostasis. Differences in SLC44A2, for instance due to variation at rs2288904, might alter the interaction between VWF and SLC44A2, possibly impacting VWF function and thereby VTE risk. Moreover, it has been shown SLC44A2 is expressed on endothelium, where it expresses an isoform involved in choline transport (17). Possibly, altered choline homeostasis affects the composition of the endothelial cell membrane and consequently the endothelium's (anti)coagulant surface. In conclusion, despite the negative outcome of the present study, the association between *SLC44A2* and VTE remains of interest for future studies.

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