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Original Article
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Dysfunction of complement receptors CR3 (CD11b/18) and CR4 (CD11c/18) in pre-eclampsia: a genetic and functional study

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Objective To study genetic variants and their function within genes coding for complement receptors in pre-eclampsia.

Design A case–control study.

Setting Pre-eclampsia is a common vascular disease of pregnancy. The clearance of placenta-derived material is one of the functions of the complement system in pregnancy.

Population We genotyped 500 women with pre-eclamptic pregnancies and 190 pregnant women without pre-eclampsia, as controls, from the FINNPEC cohort, and 122 women with pre-eclamptic pregnancies and 1905 controls from the national FINRISK cohort.

Methods The functional consequences of genotypes discovered by targeted exomic sequencing were explored by analysing the binding of the main ligand iC3b to mutated CR3 or CR4, which were transiently expressed on the surface of COS-1 cells.

Main outcome measures Allele frequencies were compared between pre-eclamptic pregnancies and controls in genetic studies. The functional consequences of selected variants were measured by binding assays.

Results The most significantly pre-eclampsia-linked CR3 variant M441K (P = 4.27E-4, OR = 1.401, 95% CI = 1.167–1.682) displayed a trend of increased adhesion to iC3b (P = 0.051). The CR4 variant A251T was found to enhance the adhesion of CR4 to iC3b, whereas W48R resulted in a decrease of the binding of CR4 to iC3b.

Conclusions Results suggest that changes in complement-facilitated phagocytosis are associated with pre-eclampsia. Further studies are needed to ascertain whether aberrant CR3 and CR4 activity leads to altered pro- and anti-inflammatory cytokine responses in individuals carrying the associated variants, and the role of these receptors in pre-eclampsia pathogenesis.

Keywords β2-integrins, complement receptors, complement system, genetic association, pre-eclampsia, pregnancy.

Tweetable abstract Genetic variants of complement receptors CR3 and CR4 have functional consequences that are associated with pre-eclampsia.

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Introduction

Pre-eclampsia is a common pregnancy-specific vascular disorder that affects 3% of pregnancies.[1] It accounts for over 50 000 maternal and 900 000 perinatal deaths annually.[2,3] The clinical characteristics are diverse, and the course of the disease is unpredictable.

Epidemiological evidence indicates that pre-eclampsia is partially inherited.[4–9] Among immunological pathways, abnormalities in the complement system have recently been found to be one of the contributing mechanisms in pre-eclampsia.[10–12] The complement system discriminates between self and non-self structures, causes inflammation, cell death and tissue destruction, and initiates adaptive immune responses. Inadequate regulation of the complement system may result in poor placentation and predispose the pregnancy to pre-eclampsia.[13,14] Three pathways of complement activation lead to a common end point: the activation of C3 and the terminal pathway, and the formation of the membrane attack complex (MAC). Genetic polymorphisms within genes coding for components of the complement system have been linked to pre-eclampsia susceptibility.[15,16] The levels of C3 are increased in inflammation but decreased or overactivated in immune diseases such as systemic lupus erythematosus (SLE), which is known to predispose women to pre-eclampsia.

When complement is activated, the generated opsonins C3b and C4b bind covalently to the targets. As activation of C3 is a critical step in complement activation, it must be controlled carefully. An important outcome of C3 inactivation is the generation of iC3b molecules, because they are recognized by complement receptors type 3 (CR3, CD11b/18, Mac-1 or integrin αMβ2) and type 4 (CR4, CD11c/18, p150.95 or integrin αXβ2). CR3 and CR4 are β2-integrins that function as complement receptors and specifically recognize iC3b.[17,18] Microbes and particles coated with iC3b are efficiently phagocytosed and eradicated by neutrophils and macrophages. In the absence of alarm signals indicating infection or major tissue injury, the iC3b-mediated phagocytosis occurs in a relatively silent fashion as a basic homeostatic clearance process.

As a result of the rapid growth of the placenta and direct exposure to maternal blood, it releases microparticles into the maternal circulation. We have hypothesized that in pre-eclampsia, a major disorder of pregnancy, the clearance of the placental particles, cells or their remnants could be abnormal. Therefore, we have studied the genetic associations of genes coding for receptors of the complement system with pre-eclampsia in a large patient cohort. β2-Integrins may play an important role in mediating the phagocytosis of particles, e.g. from damaged endothelium or placenta, as well as for their ability to control the inflammatory balance of the immune response.

Methods

Subjects and sequencing protocol

Briefly, we studied 500 women with pre-eclamptic pregnancies, who were not obese (with a body mass index of <30 kg/m^2), and 190 pregnant women without pre-eclampsia, as controls, from the Finnish Genetics of Pre-eclampsia Consortium (FINNPEC) cohort.[19] Data were combined with additional patients and controls from the national FINRISK study cohort to comprise altogether 609 cases and 2092 controls for association analyses (for details, see Table S1). Patients were not involved in the research effort. A predefined core outcome set was not used in the study design.

In a previously described custom-made targeted exomic sequencing protocol,[20] we combined an improved Illumina sequencing library for capture and sequencing with NimbleGen Sequence Capture to study variants within exons of 11 genes coding for receptors of the complement system: *ITGAM* (coding for subunit of CR3), *ITGAX* (coding for subunit of CR4), *CD93*, *C3AR1*, *ITGB2*, *C5AR1*, *C5AR2*, *C1qR*, *CR2*, *CR1* and *CR1L*. A detailed description of the study materials and methods are available elsewhere.[20]

Functional studies

To study how variants in the CR3 and CR4 receptors related to pre-eclampsia affect cell adhesion to iC3b, we transiently transfected COS-1 cells with either the wild-type β2-integrin (wt-CR3 or wt-CR4) or one of the mutant variants (M441K-CR3 and T1000N-CR3 or W48R-CR4 and A251T-CR4). Expression was established by flow cytometry and adhesion analyses were conducted according to a previously published protocol (for details, see Appendix S1).[21]

In silico studies

Loss-of-function vulnerability score per gene and PolyPhen and Sift functional *in silico* analyses per variant were conducted online in VARIANT EFFECT PREDICTOR (VEP; https://www.ensembl.org/Tools/VEP), with the following annotations: LoF score of <0.2, probably damaging; LoF score of 0.2–0.7, possibly damaging; and LoF score of >0.7, benign.

Modelling of CD11b structure

The CR3 structure was modelled on the basis of the previously solved CR4 structure (PDB: 3K71, chain G[22]) using the

PRIME module of SCHRÖDINGER SUITES 2018-4, (Schrödinger, LLC, New York, NY, USA) via the MAESTRO interface.[23,24] The generated model of CD11b was checked manually, especially for non-similar residues in comparison to CR4. The model was also verified by checking its Ramachandran diagram.

Statistical methods

Genes with variants associated with pre-eclampsia are listed in Table 1. Data were analysed with PLINKSEQ, PLINK and R.[25] The significance of the allelic associations was analysed by Fisher's exact test. Analysis was divided between low-frequency (minor allele frequency, MAF < 10%) and common (MAF > 10%) variants. KAVIAR and VEP 37 were used for additional annotations.[26,27] In addition to the appropriate statistical probability test, odds ratios (ORs) with 95% confidence intervals (95% CIs) were calculated for all variants. The Student's *t*-test was used for statistical analysis of the functional assays, and a probability value smaller than 0.05 was considered significant.

Results

Two predisposing variants in *ITGAM*, coding for the α-chain of the complement receptor type 3 (CR3, CD11b), rs41321249 (T1000N; $P = 0.044$) and rs1143680 (M441K; $P = 4.27E\text{-}4$), were found to be associated with pre-eclampsia susceptibility by unadjusted association analyses. As shown in Table 1, the more significant of these alleles, M441K, was found in 15.4% of women with pre-eclampsia, as opposed to 11.5% of the controls.

For *ITGAX* coding for the α-chain of the complement receptor type 4 (CR4, CD11c), three predisposing variants, rs17853815 (E547K; $P = 0.032$), rs2230424 (W48R; $P = 2.76E\text{-}4$) and rs2230428 (A251T; $P = 3.47E\text{-}5$), were found to be associated with pre-eclampsia susceptibility. Of these, A251T was found in 20.9% of patients versus 15.7% of controls and W48R was found in 15.3% of patients versus 11.5% of controls.

Another missense functional variant, rs201463658, was found in *CR2* coding for the complement receptor type 2 (CD21) ($P = 0.050$, possibly predisposing). This causes the change S381R, which was found to be 'probably damaging' by PolyPhen and deleterious by Sift in the *in silico* functional analyses. All significant results of the association analyses are listed in Table 1. All associated variants conformed to the proportions of Hardy–Weinberg equilibrium (HWE, $P > 0.05$). With mid-range LOFTOOL scores of 0.543 and 0.441 in the 'possibly damaging' category, the *ITGAM* and *ITGAX* genes were deemed to be somewhat resistant to the deleterious effects of variants.

The tail of the *P*-value distribution of benign variants was as expected, suggesting that the overall study design and quality control were successful. Half of the associated variants are located in genes coding the β2-integrins, and the most promising of these variants were subjected to functional studies.

Functional analyses of β2-integrin missense variants related to pre-eclampsia

The CR3 and CR4 integrins are both heterodimeric receptors consisting of a common β-chain (β2- or CD18) pairing with different α-chains: CD11b for CR3 and CD11c for CR4, respectively (Figure 1A,B). The primary protein domains of CR3 and CR4 and the relative locations of the PE-associated missense variants are shown in schematic pictures in Figure 1C, D, whereas the locations of the studied mutations in the structures of CR3 and CR4 are shown in Figure 2A, B.

To analyse the functional effects of the pre-eclampsia-associated CR3 and CR4 variants, COS-1 cells were

Table 1. Associating variants within genes coding for complement receptors in pre-eclampsia

RSID	Gene name	*P* value	OR (95% CI)	MAF (pre-eclampsia)	MAF (control)	HWE	Consequence (distance from exon, base pairs)	LoFtool*
rs2230428	*ITGAX*	3.47E-5	1.416 (1.205–1.664)	0.209	0.157	1	Missense variant, A251T	0.441
rs1143680	*ITGAM*	4.27E-4	1.401 (1.167–1.682)	0.154	0.115	0.645	Missense variant, M441K	0.543
rs2230424	*ITGAX*	2.76E-4	1.414 (1.177–1.698)	0.153	0.113	0.643	Missense variant, W48R	0.441
rs55865320	*ITGB2*	0.019	1.256 (1.041–1.516)	0.141	0.116	1	Intronic variant (−11)	0.033
rs201176761	*ITGAX*	0.019	5.634 (1.094–36.328)	0.004	<0.001	1	Intronic variant (−13)	0.441
rs61734513	*CR1*	0.027	2.824 (1.032–7.515)	0.007	0.003	1	Synonymous variant, C1907C	na
rs17853815	*ITGAX*	0.032	4.311 (0.926–21.758)	0.004	<0.001	1	Missense variant, E547K	0.441
rs2230528	*ITGB2*	0.036	1.192 (1.013–1.403)	0.200	0.170	0.904	Synonymous variant, G273G	0.033
rs41321249	*ITGAM*	0.044	1.639 (1.024–2.626)	0.022	0.013	0.348	Missense variant, T1000N	0.543
rs41258244	*CD46*	0.047	1.368 (0.995–1.863)	0.051	0.038	0.083	Intronic variant (−41)	0.983
rs201463658	*CR2*	0.050	4.596 (0.777–31.418)	0.003	<0.001	1	Missense variant, S381R	0.952

LoF, loss-of-function score from VARIANT EFFECT PREDICTOR (VEP, https://www.ensembl.org/Tools/VEP) indicates the per-gene susceptibility to disease based on the ratio of loss of function to synonymous mutations in ExAC data; MAF, minor allele frequency; RSID, reference single-nucleotide polymorphism (SNP) cluster ID.
*LoF score of <0.2, probably damaging; LoF score of 0.2–0.7, possibly damaging; LoF score of >0.7, benign.

transiently transfected with the variant and wild-type integrins. As analysed by fluorescence-activated cell sorting (FACS), all the CR3 and CR4 variants were found to be expressed at equal quantities on the surface of COS-1 cells (Figures 3A and 4A). According to this result, none of these pre-eclampsia-related CR3 and CR4 missense variants altered the integrin epitopes in such a way that would prevent the binding of the β2-integrin antibodies used in our FACS analysis. Subsequently, COS-1-transfectants were allowed to adhere to the iC3b ligand coated on plastic. Cell binding of the mutant variants and wild-type CR3 or CR4 to iC3b is shown in Figures 2B and 3B. The change of methionine 441 to lysine in CD11b resulted in a trend of increased cell adhesion of unstimulated and PDBu-stimulated M441K-CR3 transfected COS-1 cells to iC3b. A similar trend was observed in the iC3b adhesion of threonine to asparagine mutation at position 1000 (T1000N) in the Calf-2 domain of CR3 (Figure 3B).

The mutation of tryptophan 48 to arginine in CR4 (W48R) decreased the binding of either untreated or PDBu-activated CR4-transfected COS-1 cells to iC3b (Figure 4B). In contrast, the mutation of alanine 251 to threonine (A251T) in the CD11c chain increased the binding of PDBu-activated COS-1 cells to iC3b compared with wt-CD11c (Figure 4B). For unstimulated cells, the difference in binding between the mutant and wild-type CR4 was not significant.

Discussion

Main findings: amino acid variations in CR3 and CR4 affect iC3b binding

We have discovered that two missense variants in *ITGAM* coding for CR3 and three missense variants in *ITGAX*

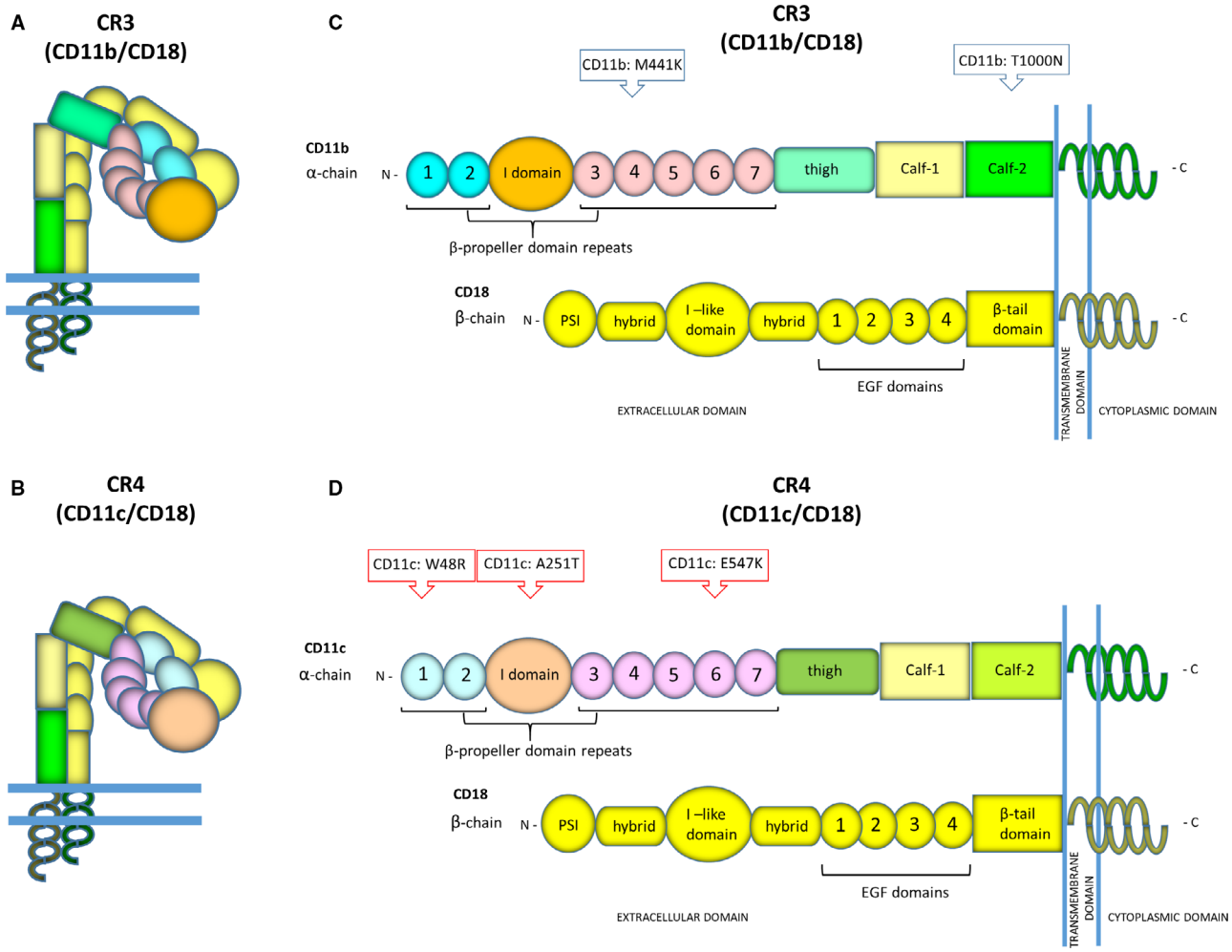

Figure 1. The relative locations of the pre-eclampsia-associated missense variants in CR3 or CR4. The complement receptors CR3 and CR4 are heterodimeric integrin-type receptors comprising α-chains, CD11b for CR3 or CD11c for CR4, that are associated non-covalently with a common β-chain, CD18. CR3 (A) and CR4 (B) are shown in their inactive and bent forms. The schematic pictures of the primary protein domains in the α-chains of the β2-integrins show the locations of CR3-related mutations (M441K, T1000N) (C) and the CR4-related mutations (W48R, A251T, E547K) (D).

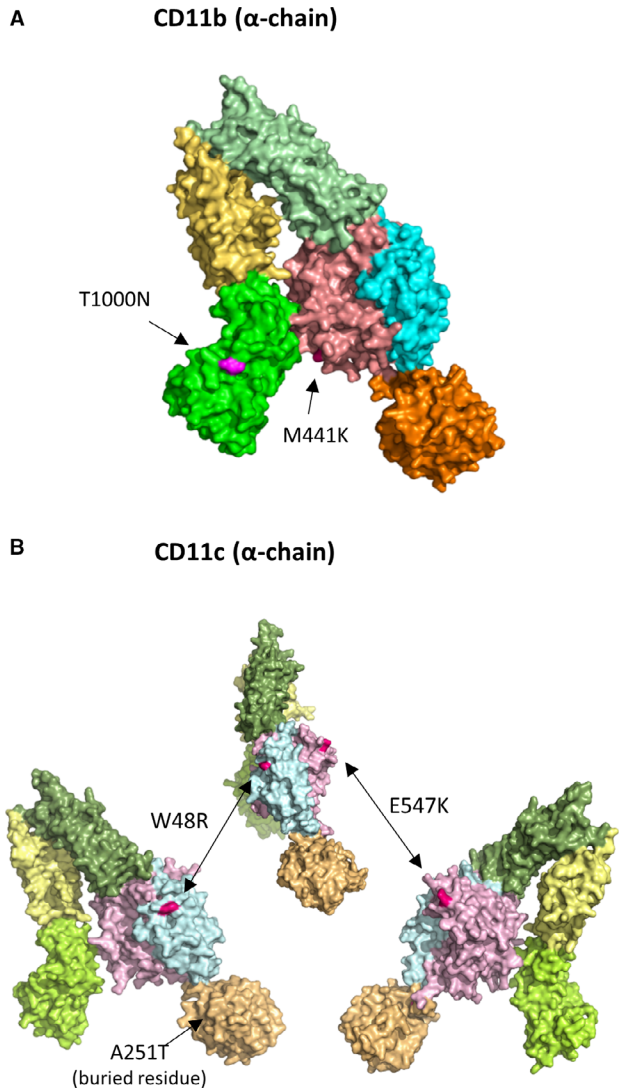

Figure 2. Locations of the pre-eclampsia-associated missense variants shown in three-dimensional structural models of CR3 and CR4. Structural models of CD11b (A) and CD11c (B) indicate the locations of pre-eclampsia-related variants (based on the crystal structure of CR4[22]). CR4 is presented from three angles to illustrate the locations of W48R and E547K variants on opposite sides of the β-propeller domain. Small arrows point to the positions of the mutations.

coding for CR4 predispose pregnant women to pre-eclampsia. The two variants with strongest association with pre-eclampsia in each gene were found to have varying functional effects on the respective protein.

The M441K variant in the fourth blade of the β-propeller domain seems to lead to a more adhering form of the integrin to iC3b, although this result was not statistically significant and thus warrants further investigation. M441K is a common variant, with a minor allele frequency (MAF) of approximately 15%, that strongly predisposes pregnant women to pre-eclampsia. Another CR3 variant, M441T (rs1143680), was shown not to affect phagocytosis of iC3b-coated sheep RBC by CR3-expressing COS-7 cells[28]. This may reflect the requirement for a positively charged amino acid at this position for the increased adhesion to iC3b.

In accordance with M441K-CR3, the variant A251T located in the iC3b-binding I-domain of CR4 was found to increase adhesion to iC3b (Figure 4B).[18] 251T, a common variant (MAF = 20%), is associated with pre-eclampsia with the most robust probability value among the associations discovered in complement receptor genes. The stronger adhesion of 251T-CR4 to iC3b, like 441K-CR3, may result in a shift to a more inflammatory CR4-mediated response.

In contrast to the above-mentioned variants, the variant W48R of CR4, located in the first blade of the β-propeller domain, resulted in a decrease in its binding capacity to iC3b. As the binding site for iC3b in CR4 appears to be in the I-domain, and no binding sites in the beta-propeller have been described,[18] the W48R effect on adhesion to iC3b is probably indirect and may affect the binding of several ligands. Similar diminished adhesion to iC3b as observed with W48R on CR4 has previously been shown with the R77H-substituted CR3, which is associated with SLE.[29] The R77H mutation is located in the second blade of the β-propeller domain of CR3. In a pregnant woman, SLE is known to increase the risk of pre-eclampsia between two- and four-fold.[30]

Variants were observed as heterozygous mutations with the exception of one individual, who was found to be homozygous for the 1000N variant in ITGAM coding for CR3. She was diagnosed with severe late-onset pre-eclampsia complicated by HELLP syndrome (haemolysis, elevated liver enzymes and low platelet count) postpartum.

We found that the T1000N predisposing variant of CR3 adhered to iC3b similarly as the wild-type CR3 integrin or may possibly have an increasing effect. The implications of T1000N are discussed in Appendix S2.[15,18,31-34]

Strengths and limitations

The FINNPEC cohort is exhaustively characterized and allows for specific clinical phenotyping with severe disease, which is a strength in our study. None of the observed variants were unique to the patients and they are therefore unlikely to be the only causative factor of pre-eclampsia in our cohort. Although iC3b is the main ligand for β2-integrins, the interactions with other ligands were not studied.

Interpretations

The important phagocytic complement receptors CR3 and CR4 belong to the family of β2-integrins, surface receptors of leukocytes that play critical roles in innate and adaptive immune responses. Human CR3 and CR4 share significant similarity: they are 87% homologous according to the

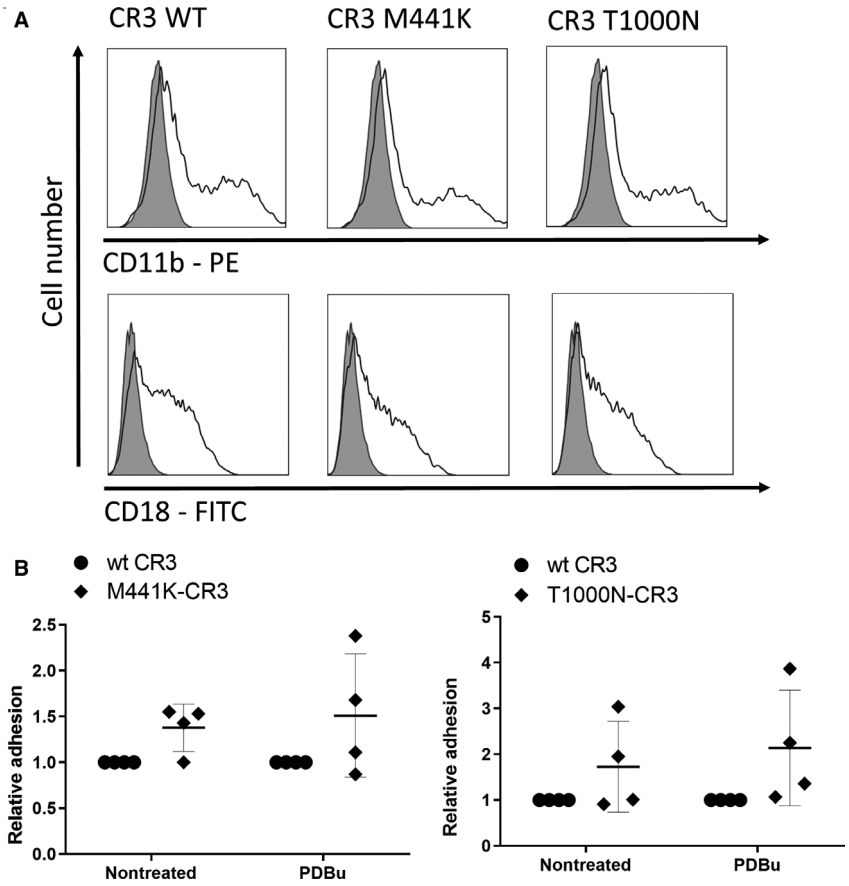

Figure 3. The CR3 mutant M441K, located in the β-propeller domain, adhered more strongly to iC3b than the wild-type CR3 integrin, whereas no difference in binding between the CR3 mutant T1000N and the wild type was observed. The latter residue is located in the membrane proximal area. (A) Representative flow cytometry histograms show the equal expression of CD11b and CD18 between the wild-type (wt) and mutant CR3-transfected COS-1 cells. Shaded areas, non-transfected cells; solid lines, transfected cells. (B) Non-treated or PDBu-activated COS-1 cells transfected with wt-CD11b/CD18 or M441K-CD11b/CD18 or T1000N-CD11b/CD18 were allowed to bind to iC3b coated on plastic, and bound cells were detected enzymatically. Cell binding is reported relative to wt-CD11b/CD18 transfectant binding, with wt values defined as 1. Data are from three or four independent experiments. Error bars represent SDs and horizontal lines show the mean values. $*P < 0.05$.

sequence analysis of their encoding cDNAs.[35,36] However, they bind to distinct sites on iC3b.[18] CR3 and CR4 are predominantly expressed on myeloid cells, including neutrophilic granulocytes, monocytes, macrophages and dendritic cells, but are also found on B- and T-lymphocytes and lymphoid natural killer (NK) cells.[37] Although both integrins are expressed by similar cell types, their patterns and functions can differ.[38–40] CR3 and CR4 have been reported to bind to many of the same ligands, including iC3b, ICAM-1 and fibrinogen.[37,41]

We propose that the genetic variants in CR3 and CR4 may affect the ability of the maternal system to respond to placental or endothelial injury or could influence other interactions that these receptors may have during pregnancy. For example, changes in the ability to clear placental debris from the maternal circulation may alter waste removal and consequent inflammatory or coagulation system homeostasis during pregnancy. A key causative mechanism of early-onset pre-eclampsia is suggested to be the shedding of fragmented syncytiotrophoblast particles from the placenta into the maternal circulation.[42] Accumulating material in blood vessels and kidney capillaries could thus increase vascular resistance and lead to increased blood pressure and kidney dysfunction, with consequent proteinuria.[43,44] A correlation of complement activation and antiangiogenic activity has been established, although it remains unclear whether complement activation is the cause or the consequence of the antiangiogenic balance observed in pre-eclampsia.[45]

The phagocytosis of placental material is assisted by blood opsonins, such as the complement component iC3b, which is recognized by the CR3 and CR4 receptors on phagocytic cells.[46,47,48] Opsonization helps the body to maintain homeostasis and avoid further tissue damage. Apoptotic cells opsonized by iC3b can be ingested by macrophages and dendritic cells through CR3 and CR4, without triggering inflammatory responses such as the release of oxygen radicals or pro-

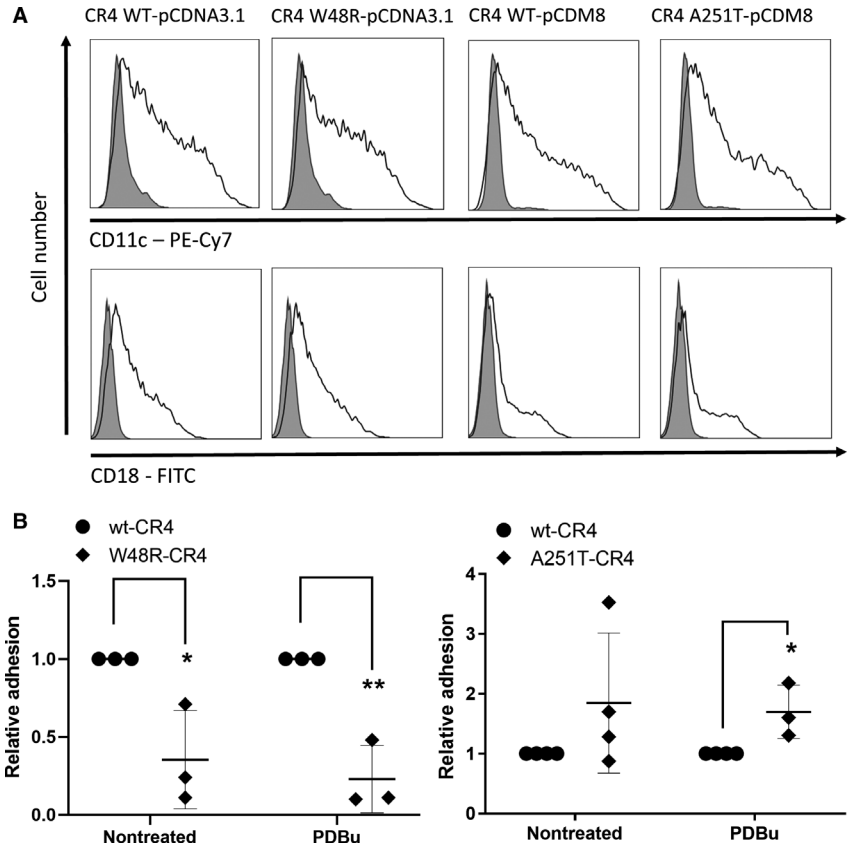

Figure 4. W48R mutation in the β-propeller area decreases and A251T mutation in the I domain increases the adhesion of CR4-integrin to complement component iC3b. (A) Representative histograms of flow cytometry analysis show an equal expression of CD11c/CD18 between the wild-type (wt) and mutant CR4-transfected COS-1 cells. Shaded areas, non-transfected cells; solid lines, transfected cells. (B) Non-treated or PDBu-activated COS-1 cells transfected with wt-CD11c/CD18, W48R-CD11c/CD18 or A251T-CD11c/CD18 were allowed to bind to iC3b coated on plastic, and bound cells were detected enzymatically. Cell binding is reported relative to wt-CD11c/CD18 transfectant binding, with wt values defined as 1. Data are from three or four independent experiments. Error bars represent SDs and horizontal lines show the mean values. *$P < 0.05$.

inflammatory cytokines.[49–52] β2-Integrins also mediate interactions between immune cells, including the formation of the immunological synapse between the T-cell and the antigen-presenting cell. β2-Integrins also influence pro- and anti-inflammatory signalling pathways of leukocytes and affect cytokine production.[40] β2-Integrins promote leukocyte extravasation by facilitating interactions between the leukocytes and endothelium. Leukocyte–endothelial interaction is increased in pre-eclampsia.[53]

The engagement of CR3 by iC3b on macrophages may result in immune-inhibitory responses, such as the production of anti-inflammatory cytokines IL-10 and TGF-β that may block the function of NF-κB, the transcription factor needed for the promotion of the transcription of many pro-inflammatory genes.[54] Increased serum levels of IL-10 have been reported in the third trimester of pre-eclamptic pregnancies.[55] However, CR3 can also have strong inflammatory effects in cell neutrophils and monocytes.[40,56,57,58]

Women with small-for-gestational-age neonates have a significantly higher expression of CR3 in their peripheral blood granulocytes and monocytes than is normal for pregnant women. Interestingly, slightly elevated expression levels of CR3 have been observed in granulocytes in women with pre-eclampsia.[59] Furthermore, a significantly higher median mean channel brightness (MCB) of CR3 and lower MCB of CD62L in granulocytes, and high MCB of CR3 in monocytes, in association with oxidative burst and basal intracellular reactive oxygen species (iROS) concentration, have been observed in women with pre-eclampsia.[60] In contrast, CR4-expressing monocytes have been observed in normal quantities in the peripheral blood of women with pre-eclampsia.[61] CR4 contributes to pro-inflammatory functions of monocytes, macrophages and dendritic cells.

Conclusion

It is likely that pre-eclampsia develops as a result of multiple genetic and/or environmental factors. Our results reflect the complex roles of CR3 and CR4 in downstream signalling and leukocyte function, because β-integrins are

clearly involved in both pro-inflammatory and anti-inflammatory effects. Future research will decipher whether disturbances in the functions of integrins, such as those reported here, contribute to a shift towards a more pro-inflammatory immune response, and whether the phenomenon is observed in non-severe as well as severe pre-eclampsia. On the other hand, in discordant cases a disruption in the function of these key receptors may result in the dysregulation of inflammation in early pregnancy or, perhaps more importantly, the clearance of placental particles or trophoblast cells during late pregnancy.

Disclosure of interests

AIL reports personal fees from Alexion, outside the submitted work. JES reports personal fees from ReAlta Life Sciences, grants from UCB and personal fees from UCB, outside the submitted work. The remaining authors have no disclosure of interests. Completed disclosure of interests forms are available to view online as supporting information.

Contribution to authorship

The genetic study was planned by JPA, HL and JES, and carried out by MT, MD, ED, AIL and MIK. MP and KA provided data from the FINRISK samples, whereas the initial targeted exomic sequencing was carried out in FINNPEC samples. SM and SF planned the functional studies, which were carried out by LT, LU and MLA. *In silico* analyses were carried out by AIL and AB. AIL drafted the first manuscript with help from LT. All authors contributed to and approved the final version of the article for publication.

Details of ethical approval

All subjects provided written informed consent and the FINNPEC study protocol was approved by the coordinating Ethics Committee of the Hospital District of Helsinki and Uusimaa (permit number 149/E0/07). The National Finrisk cohort was accessed by FINRISK licence #8/2016. The National FINRISK Study description and ethical approvals are available online: https://thl.fi/documents/10531/862648/2016+FINRISK+description_for_researchers.pdf/aaac6110-170d-4008-83e9-930262c5a868.

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Data availability statement

Data available on request from the authors.

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. Clinical characteristics of targeted exome sequencing participants.

Appendix S1. Supplementary material. Details of the patient cohorts and laboratory methods of functional studies.

Appendix S2. Supplementary information on T1000N variant in CR3.

Video S1. Author Insights. ■

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