polyclonal antisera[29], followed by horseradish peroxidase-conjugated protein A. Lysates were immunoprecipitated with either 5 μg anti-HA 12CA5 or 3 μg anti-Myc 9E10 monoclonal antibody for 2 h at 4 °C, followed by incubation with protein G–Sepharose for 1 h at 4 °C. For pulse-chase analysis, cells were labelled for 30 min with ^{35}S-methionine/cysteine, chased in medium with excess unlabelled methionine/cysteine for varying times and collected by detergent lysis. After immunoprecipitation and SDS–PAGE, gels were visualized by fluorography and quantified by densitometry (Molecular Analysis, Biorad). For subcellular fractionation, adherent cells were detached by incubation with EDTA and gently disrupted by Dounce homogenization. Cellular lysates were layered on 45% sucrose cushions and membrane and cytosolic/microsomal fractions recovered after centrifugation at 7,000*g* for 20 min.

Received 16 January; accepted 12 May 1997.

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Acknowledgements. We thank G. Robertson for HERG cDNA, J. Nerbonne and A. Pond for HERG antisera, K. Sawada for E4031, and V. S. Srinivas for technical assistance. This work was supported by funds from the AECOM Molecular Cardiology Endowment to T.V.M., M.B.M. and G.I.F., and by grants to G.I.F. from the American Heart Association (Established Investigator) and the Council for Tobacco Research and to S.A.N.G. from the NIH-NIGMS and Donaghue Foundation.

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P. falciparum rosetting mediated by a parasite-variant erythrocyte membrane protein and complement-receptor 1

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The factors determining disease severity in malaria are complex and include host polymorphisms, acquired immunity and parasite virulence[1]. Studies in Africa have shown that severe malaria is associated with the ability of erythrocytes infected with the parasite *Plasmodium falciparum* to bind uninfected erythrocytes and form rosettes[2–5]. The molecular basis of rosetting is not well understood, although a group of low-molecular-mass proteins called rosettins have been described as potential parasite ligands[6]. Infected erythrocytes also bind to endothelial cells, and this interaction is mediated by the parasite-derived variant erythrocyte membrane protein PfEMP1 (refs 7, 8), which is encoded by the *var* gene family[9–11]. Here we report that the parasite ligand for rosetting in a *P. falciparum* clone is PfEMP1, encoded by a specific *var* gene. We also report that complement-receptor 1 (CR1) on erythrocytes plays a role in the formation of rosettes and that erythrocytes with a common African CR1 polymorphism (Sl(a$^-$))[12] have reduced adhesion to the domain of PfEMP1 that binds normal erythrocytes. Thus we describe a new adhesive function for PfEMP1 and raise the possibility that CR1 polymorphisms in Africans that influence the interaction between erythrocytes and PfEMP1 may protect against severe malaria.

We studied the role of PfEMP1 in rosetting using the clone R29 of *P. falciparum*, which was divided into isogeneic rosetting (R29R$^+$) and non-rosetting (R29R$^-$) subpopulations by sedimentation in gelatin[13] (see Methods). Each parasite genome is estimated to contain 50–150 *var* genes[11], and switching of expression from one *var* to another gives rise to antigenic variation[14]. To study the *var* genes expressed by R29R$^+$ and R29R$^-$, we used the polymerase chain reaction with reverse transcription (RT-PCR) and degenerate oligonucleotide primers to the first cysteine-rich Duffy-binding-like domain (DBL-1) of the *var* genes. This DBL-1 domain of known *var* genes has regions of very high sequence homology interspersed with regions of variable sequence[11]. The RT-PCR product from R29R$^+$ RNA consisted of a single major band, whereas three distinct bands were visible from R29R$^-$ RNA (Fig. 1a). RT-PCR products were cloned into a pCRII vector and those with inserts of the appropriate size for DBL-1 (400–600 base pairs (bp)) were sequenced. From the cloning of the R29R$^+$ RT-PCR product, 13 recombinant plasmids contained inserts of 400–600 base pairs. These all contained the DBL-1 sequence (called *R29R$^+$var1*; Fig. 1b), which is identical to the *var* sequence described for the R29 clone[10]. From the cloning of the R29R$^-$ RT-PCR product, five different DBL-1 sequences were detected, namely *R29R$^-$var2* to *R29R$^-$var6* (Fig. 1b). None of the recombinant plasmids from the R29R$^-$ RT-PCR product contained the *R29R$^+$var* sequence found in rosetting parasites. Using primers specific for *R29R$^+$var1*, we confirmed by RT-PCR that this gene is specific to the R29R$^+$ population and is not expressed by the R29R$^-$

non-rosetting parasites (Fig. 1c). The presence of amplifiable RNA in the R29R$^-$ sample was demonstrated by RT-PCR with specific primers to *R29R$^-$var3* which gave a 550-bp band with R29R$^-$ RNA (Fig. 1c). Thus, a particular *var* gene, *R29R$^+$var1*, is expressed by rosetting parasites but not by non-rosetting parasites of the R29 clone. This finding is consistent with a role for *var*/PfEMP1 in rosetting.

To examine the functional properties of *R29R$^+$var1*, the sequence was extended by PCR-walking using vectorette libraries[15]. We obtained seven overlapping clones encoding all of exon 1 (the entire extracellular domain and the transmembrane region), the intron, and the 5′ half of exon 2 (the acidic intracellular domain). The clones were sequenced in both directions and gave an open reading frame of 8.2 kilobases (kb). *R29R$^+$var1* has the characteristic *var* gene structure[9,11], with the extracellular portion consisting of four DBL domains and a cysteine-rich interdomain region (CIDR) (Fig. 1d).

To study the binding properties of *R29R$^+$var1* to red blood cells (RBCs), constructs were made to express individual DBL domains and the CIDR in COS-7 cells as chimaeric proteins with the herpes simplex virus glycoprotein D[16]. COS-7 cells were transiently transfected, and surface expression of DBL domains was determined by immunofluorescence assay with monoclonal antibodies against the herpes simplex glycoprotein D[17]. Each domain of the *R29R$^+$var1* gene was expressed on the surface of COS-7 cells except DBL-4 (Fig. 1d). The transfected COS-7 cells were tested for their ability to bind RBC. DBL-1 of *R29R$^+$var1* bound RBC, whereas DBL-2, DBL-3, and CIDR did not (Fig. 1d, e). As evidence that not all DBL-1 domains bind RBC, DBL-1 expressed by a non-rosetting parasite (A4 (ref. 10); gift from J. Smith) was expressed on ~2% of COS-7 cells but did not bind RBC.

We studied the effect of the anti-CD36 monoclonal antibody OKM5 on RBC binding to DBL-1 of *R29R$^+$var1* because PfEMP1 is known to bind to CD36 (refs 7, 8) and previous work has shown that CD36 is expressed at low levels on RBC and can rarely act as a rosetting receptor[18]. OKM5 (100 μg ml^{-1}) did not inhibit RBC binding to DBL-1 of *R29R$^+$var1* expressed in COS-7 cells (36 positive cells with OKM5, compared with 32 positive cells with an isotype-matched control; mean binding in 50 fields at 200× magnification from two experiments). R29 rosetting also is not inhibited by OKM5 (ref. 19), so CD36 binding does not play a role in the interaction between PfEMP1 and RBC described here.

Confirmation that binding of RBC to DBL-1 is the basis of rosetting was hampered by the fact that the RBC receptor for R29 was unknown. We therefore tested 23 RBC variants that were negative or null for a range of high-frequency blood group antigens to determine whether any of these RBC were unable to form rosettes with five *P. falciparum* clones/lines. Initial experiments were with frozen RBC, and the experiments were repeated with fresh RBC for any variant that showed reduced rosetting. The Knops null RBC was the only one to show consistently reduced rosetting (Fig. 2a). Knops null RBC express low copy numbers of complement-receptor 1, having fewer than 100 CR1 molecules per RBC compared with the normal range of 100 to 800 (refs 20, 21). Previous work had shown that the ABO blood grouping influences the size of rosettes formed but not the overall rosette frequency[22]. Our results, however, were not dependent on the ABO blood group of the RBC. To exclude any artefact resulting from the purification of infected RBC for the rosette-reformation assay, fresh CR1-deficient RBC were obtained from another donor (B.S.; CR1 copy number 60), and the rosetting R29R$^+$ parasites were cultured *in vitro* for 9 days in the CR1-deficient and control normal RBC. Blinded assessments of rosette frequency were made every cycle, and the reduced rosetting of CR1-deficient RBC was confirmed (Fig. 2b). The parasitaemia in CR1-deficient RBC was equivalent to the control, and Giemsa-stained smears showed that the parasites were morphologically normal. We have therefore demonstrated reduced rosetting of CR1-deficient RBC from four different donors (two frozen and two fresh) either by rosette reformation assay or by continuous culture.

Our results suggest that CR1 plays a role in the formation of rosettes. We therefore examined the effect of soluble recombinant CR1 (sCR1; ref. 23) on rosetting. sCR1 inhibited rosette formation in three out of four parasite clones/lines tested (Fig. 2c, d). These

Table 1 Binding of CR1-deficient RBC to DBL-1 of *R29R$^+$var1* expressed in COS-7 cells

Donor	RBC binding*
CR1-deficient (frozen)	0
Control	27
CR1-deficient (M.H.)	0
Control	45
CR1-deficient (B.S.)	7
Control	22

* Number of positive COS-7 cells in 50 fields at 200× magnification. Data shown represent the mean binding from two independent experiments for each sample.

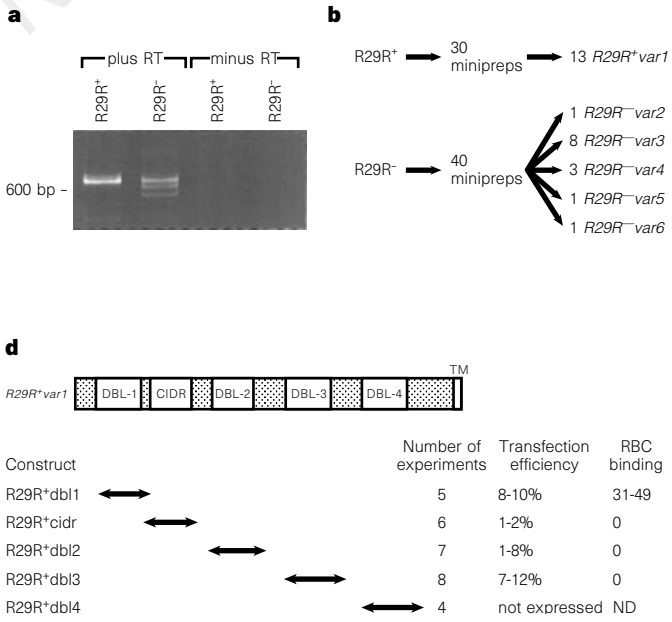

Figure 1 *Var* gene expression and RBC binding in *P. falciparum* clone R29. **a**, RT-PCR analysis of *var* gene expression in rosetting (R29R$^+$) and non-rosetting (R29R$^-$). To control for possible genomic DNA contamination, reactions with and without reverse transcriptase (RT) were performed in parallel. **b**, Cloning and sequence of RT-PCR products from **a**. All minipreps with inserts of 400–600 bp were sequenced. **c**, RT-PCR analysis with specific primers to *R29R$^+$var1* (R$^+$ primers) and *R29R$^-$var3* (R$^-$ primers). IRBC, infected RBC. **d**, Expression constructs and RBC binding of domains of *R29R$^+$var1*. ND, not determined. **e**, RBC binding to COS-7 cell transiently transfected with DBL-1 of *R29R$^+$var1*.

results are consistent with the importance of CR1 in the formation of rosettes. The reason for the failure of sCR1 to inhibit rosetting by PAR^+ is unknown, although it may relate to additional receptors or affinity of PAR^+ for sCR1.

CR1-deficient RBC were used to determine the specificity of RBC binding to DBL-1 of *R29R*$^+$*var1*. CR1-deficient RBC from three donors showed reduced or absent binding to DBL-1 compared with control RBC (Table 1). Thus, a particular *var* gene (*R29R*$^+$*var1*) is expressed by rosetting but not by non-rosetting parasites of the R29 clone, and DBL-1 of this *var* gene binds RBC with the same specificity as R29 rosetting parasites. Together, these data provide strong evidence that PfEMP1 encoded by *R29R*$^+$*var1* is the rosette-mediating ligand in R29.

The high mortality of *P. falciparum* in endemic areas has resulted in the selection for polymorphisms that afford protection from severe malaria[24,25]. The association between the rosetting phenotype and severe disease from *P. falciparum* suggests that natural RBC variants that support rosetting less well may have been selected to a high frequency by reducing mortality from malaria. The Sl(a$^-$) CR1 blood-group polymorphism occurs in ~30% of African Americans but is rare in Caucasians[12]. We tested a blinded panel of Sl(a$^+$) and Sl(a$^-$) RBC for their ability to bind to DBL-1 of *R29R*$^+$*var1* expressed in COS-7 cells. RBC from each donor were characterized for their CR1 copy number and Sl(a) type (Table 2). Sl(a$^-$) RBC consistently showed lower binding to DBL-1 than did Sl(a$^+$) controls ($P = 0.0001$). Confirmation that Sl(a$^-$) and another high-frequency polymorphism of CR1 in Africans (McC(b$^+$)[12] are protective alleles will require molecular definition of the polymorphisms and their evaluation in large case-control studies of severe malaria.

Rosetting is a heterogeneous phenomenon in which a number of red-cell ligands (ABO blood group[22], CD36 (ref. 18)) and soluble molecules (immunoglobulins[26], sulphated glycoconjugates[19]) differentially modify the degree of rosette formation in different isolates. Small proteins (rosettins) on the infected RBC surface have also been associated with the rosetting phenotype[6]. Multiple pathways of rosette formation may exist, but the fact that rosetting of five independent parasites is reduced in RBC with low expression of CR1 suggests that CR1 may play a common but not exclusive role

Table 2 Binding of a blinded panel of Sl(a$^+$) and Sl(a$^-$) erythrocytes to COS-7 cells expressing DBL-1 of *R29R*$^+$*var1*

Donor	Race*	CR1 copy number	Sl(a) Type	Binding† to COS-7 cells expressing DBL-1				
				Expt 1	Expt 2	Expt 3	Expt 4	Expt 5
Set 1								
960673	W	180	–	7, 8	21, 15	ND‡	ND	ND
960675	B	251	–	13, 9	14, 7	ND	ND	ND
960676	B	309	–	31, 23	38, 31	ND	ND	ND
960671	W	229	+	38, 29	56, 32	ND	ND	ND
960670	B	205	+	61, 55	72, 81	ND	ND	ND
960674	W	372	+	62, 61	76, 70	ND	ND	ND
Set 2								
960729	B	273	–	ND	19, 14	21, 36	ND	ND
960014	B	298	+	ND	47, 38	49, 66	ND	ND
920560	B	332	+	ND	70, 47	85, 87	ND	ND
960048	B	336	+	ND	71, 87	76, 123	ND	ND
Set 3								
960746	B	274	–	ND	ND	ND	0, 8, 2	11, 19, 8
960745	B	342	–	ND	ND	ND	9, 14, 7	18, 28, 11
960748	H	325	+	ND	ND	ND	53, 38, 38	53, 88, 68

* W, white; B, black; H, hispanic.
† Data shown are number of positive COS-7 cells in 50 fields at 200× magnification for two coverslips (experiments 1–3) or three coverslips (experiments 4 and 5). The binding data were subjected to a three-way analysis of variance (Sl(a) type, experiment, donor; PROC GLM procedure, SAS Institute). The difference between respective mean binding values (Sl(a$^-$) 15.8 ± 1.9 s.e. versus Sl(a$^+$) 62.3 ± 3.8 s.e.) accounted for two-thirds of the overall observed variability and was significant at the 0.0001 level (18.3, 42 degrees of freedom).
‡ ND, not determined.

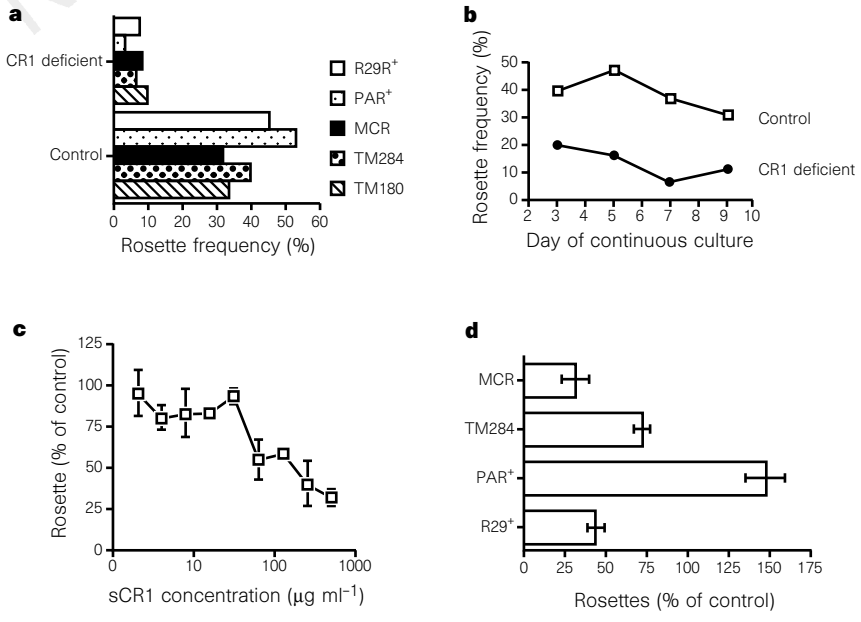

Figure 2 CR1 is required for *P. falciparum* rosetting. **a**, Rosette formation of fresh CR1-deficient RBC (donor MH; CR1 copy number, 28) with five parasite clones/lines. **b**, Rosetting of R29 grown in continuous culture in CR1-deficient (donor BS; CR1 copy number, 60) and normal RBC. **c**, Dose-dependent inhibition of R29 rosetting by sCR1. Rosetting at each sCR1 concentration is expressed as a percentage of the rosette frequency in control R29 with no added sCR1. Data shown are mean and standard error of triplicate determinations of rosette frequency at each concentration from a representative experiment. Two additional experiments gave similar results. **d**, Effect of 100 μg ml^{-1} sCR1 on four parasite clones/lines. Rosetting is expressed as a percentage of the control rosette frequency for each clone/line without added sCR1. Data shown are mean and standard error from three ($R29R^+$, PAR^+ and MCR) or four (TM284) independent experiments.

in the process and may contribute to a more complex multimolecular interaction involving PfEMP-1. Although rosetting is associated with severe disease in most studies, the present data cannot distinguish between the possibilities that rosetting itself contributes to pathology by exacerbating microvascular obstruction or that it represents a surrogate marker for some other adhesive interaction with, for example, endothelial cells. Further insight into the molecular mechanisms involved may lead to new treatments and prevention of severe *P. falciparum* infections. □

Methods

Parasite culture. Parasites were grown as described[14]. *P. falciparum* used were the clones R29 (ref. 14) and PAR$^+$ (ref. 6), and the lines MCR[18], TM180 (ref. 22), and TM284 (ref. 22).

Gelatin sedimentation. Sedimentation of parasite culture in plasmagel (3% gelatin in isotonic saline) results in separation of rosetting and non-rosetting infected RBC[13]. The upper non-rosetting fraction is completely free of rosetting infected RBC, whereas the bottom fraction is predominantly rosetting infected RBC but always contains a small proportion of non-rosetting infected RBC. The weak band from RNA of the rosetting infected RBC with R$^-$ primers (Fig. 1c) reflects this contamination of the R$^+$ fraction.

RT-PCR. RNA extraction and RT-PCR of *var* gene DBL-1 were done as described[10]: upstream primer (primer 1), 1′GC(T/C/A)TG(T/C)GCICCIT(T/A)(C/T)(C/A)G, which is specific for DBL-1; downstream primer, UNIEBP3′, 5′-CCA(A/T)C(T/G)(T/G)A(A/G)(A/G)AATTG(A/T)GG, which recognizes all DBL domains[27]. The *R29R$^+$var1*-specific primers were 5′-TGC ACC ATT CAG AAG ACA and 5′ TCC TTG ACT TGT AAA AGC. The *R29R$^-$var3*-specific primers were 5′ CGA CGT CTA CAT CTA TGT and 5′ ATT TTT CGC CTG TAG GTT. For the specific primers, 30 cycles of amplification were performed at 94 °C for 30 s, 55 °C for 30 s and 65 °C for 1 min 30 s.

COS-7 cell expression and RBC binding. Constructs for the expression of individual DBL domains as chimaeric proteins with the herpes simplex glycoprotein D signal sequence and transmembrane region were made using the pRE4 vector as described[16]. pR29R$^+$dbl1 extends between nucleotides 244–1,377 (numbered from the first ATG of *R29R$^+$var1*), pR29R$^+$cidr from 1,276–1,328, pR29R$^+$dbl2 from 2,326–3,207, pR29R$^+$dbl3 from 3,391–4,389, and pR29R$^+$dbl4 from 4,774–6,117. COS-7 cells were grown on 12-mm coverslips in 35-mm wells as described[16] and transfected with 1 μg DNA per well using Lipofectamine (Life Technologies). Surface expression was detected by immunofluorescence assay using the monoclonal antibody DL6 to the pRE4 vector[17]. Binding assays were carried out with RBC at 50% haematocrit in binding medium (RPMI without bicarbonate, with 10% human serum) for 2 h at room temperature. Coverslips were washed gently with PBS0.1% BSA to remove non-adherent RBC and viewed microscopically. A COS-7 cell was scored as positive when adherent RBC covered more than 50% of the cell surface. The number of positive cells in 50 fields at 200× magnification was counted.

Rosetting of RBC variants. Infected RBC were purified by heparin disruption of rosettes, followed by centrifugation through a Percoll gradient[13] and were stained with 20 μg ml^{-1} ethidium bromide. The test RBC were thawed on the day of the experiment and labelled with the fluorescent dye PKH26 (ref. 28). Control experiments showed that freezing and thawing and labelling with PKH26 did not impair the ability of normal RBC to form rosettes. The test RBC were mixed with purified infected RBC and incubated at room temperature for 30 min on a plate shaker at 300 r.p.m. The rosette frequency (percentage of mature infected RBC in rosettes) was assessed by fluorescence microscopy. A minimum of 100 infected RBC were counted and scored for rosetting, a rosette being defined as the binding of two or more labelled RBC. The null/negative RBC tested were: Knops null (2 donors), Kell null (Ko), Bombay, Rh null, LW (a$^-$b$^-$), McLeod, Kidd null (Jk-3), Duffy (a$^-$b$^-$), Gerbich (−1, −2, −3), Lutheran (a$^-$b$^-$), Gregory (a$^-$), JMH null, M^{k}M^k, Scianna (−1, −2), P (Tja$^-$), Colton (a$^-$b$^-$), Adult i, Chido negative, Lewis (a$^-$b$^-$), Xg negative, Diego (b$^-$), Lan negative, and Vel negative.

Rosette inhibition with sCR1. sCR1 was a gift from D. Fearon[23]. Parasite cultures were stained with ethidium bromide and washed once and resuspended at 4% haematocrit in complete RPMI with 10% heat-inactivated human serum. An equal volume of sCR1 in RPMI was added and the rosettes disrupted by passing the suspension six times through a 25-gauge needle. R29 was incubated with doubling dilutions of sCR1, giving a range of final concentrations from 500 μg ml^{-1} to 1.95 μg ml^{-1}. MCR$^+$, PAR$^+$ and TM284 were tested at 100 μg ml^{-1} of sCR1. Cultures were incubated at room temperature for 30 min, then assessed by fluorescence microscopy, with 200 infected RBC counted and scored for rosetting.

Determination of CR1 copy number and Sl(a) type. The mean CR1 copy number per RBC was determined by a double-antibody capture immunoassay using solubilized erythrocyte membranes[21]. Sl(a) type was determined by the antiglobulin technique[29] using human antisera to Sl(a).

Received 17 March; accepted 12 May 1997.

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Acknowledgements. We thank J. Proctor, D. Mallory, L. McCall and B. Smith for negative and null erythrocytes; M. Wahlgren and R. Howard for parasite clones/lines; G. Cohen and R. Eisenberg for the pRE4 plasmid and monoclonal antibodies; J. Smith for the A4var expression construct; D. Fearon for sCR1; D. Alling for statistical advice; and B. Sullivan for assistance in identifying Sl(a$^-$) donors. This work was funded by the Wellcome Trust (J.A.R. and C.I.N.), the National Blood Foundation (J.M.M.), and the Arthritis Foundation (J.M.M.).

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