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**COMPLEMENT PROTEINS AND
DECOMPRESSION SICKNESS SUSCEPTIBILITY**

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This report has been reviewed and is approved for publication.



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13. ABSTRACT (Maximum 200 words) Previous work implicated activation of complement proteins C5a and C3a by venous gas emboli (VGE) in the etiology of decompression sickness (DCS). The current effort used enzyme-immunoassay (EIA) methodology in addition to the radio-immunoassay (RIA) method previously used to determine the level of complement activation in blood plasma samples exposed to bubbles of air. Eight healthy male subjects who had histories of VGE-susceptibility when exposed to simulated altitudes above 20,000 ft were included in the study. Four subjects were DCS-resistant, and four were DCS-susceptible. Ethylene-diamine-tetraacetic acid (EDTA) plasma samples from these subjects were split into equal parts and incubated for 30 min in polypropylene tubes at 37° C. Test samples were incubated in the presence of air bubbles, and control samples were incubated without bubbles. C3a (RIA) and iC3b (EIA) in bubbled samples were activated to levels of 386 ng/ml and 21.2 µg/ml (approximately 36% more than controls; p < 0.05). Significant activation of other proteins by bubbles or zymosan may have been inhibited by lack of sufficient magnesium due to use of EDTA. Results from this limited sampling did not suggest a difference between complement activation in DCS-susceptible subjects and DCS-resistant subjects. The findings suggest a potential for use of EIA methodology in studying the effects of bubbles on human plasma and stress the need for further study to clarify the requirement for magnesium ions by proteins of the complement pathways.				
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COMPLEMENT PROTEINS AND DECOMPRESSION SICKNESS SUSCEPTIBILITY

INTRODUCTION

Research reports from Ward and his associates have indicated that decompression sickness (DCS) is mediated via activation of the complement system as a result of venous gas emboli (VGE) introduced during the ascent phase of a dive (15-23). Activation of complement leads to inflammation which can result in symptoms similar to those of DCS. If the VGE-complement relationship theorized by Ward could be validated, tests for complement activation could predict susceptibility to DCS.

Ward et al. (17,20) performed experiments with rabbits in which the alternate pathway of complement was inactivated. The experimental rabbits did not get DCS on subsequent exposure to conditions which produced DCS in controls. During another study by Ward et al. (18,20), 15 human volunteers were subjected to a series of hyperbaric profiles that were severe enough to produce Doppler-detectable VGE. The individuals identified as more sensitive to complement activation were shown to be also more susceptible to DCS (18). It was suggested by these studies that the unpredictable response to pressure changes, or intra-subject variation, can be explained by the variation in the sensitivity of their complement system to activation by VGE. Hence, a method of determining individual susceptibility to DCS could be developed by determining the response of that individual's complement system to VGE. A predictive capability for DCS would allow selection of pilots and astronauts based on DCS risk.

Two assay "kit" methods are now available for studying activation of complement proteins by bubbles. The radioimmunoassay (RIA) technique for quantifying effects of bubbles on complement proteins C3a and C5a was first used by Ward et al. (18). This method employs a rotating-tube, bubble-generation device (17). Use of an enzyme immunoassay (EIA) technique to study VGE-blood interactions has not been reported in the open literature. The EIA technique is less expensive, does not require a radioisotope-certified laboratory, is more sensitive (according to Quidel Inc.), and uses kits with a longer shelf life than the RIA technique. Since all of the published results have used RIA methods and all but one report (11) emanated from one laboratory, use of the EIA technique for this application was proposed.

The available EIA kits analyze iC3b, Bb, and SC5b-9 which are different proteins of the complement pathways than are the C3a and C5a analyzed by Dr. Ward's RIA method. Despite the differences in which complement proteins are analyzed, if results from use of the EIA method and RIA method were comparable, a relatively inexpensive and convenient substitute would be identified for further study of a possible relationship between VGE and DCS. If the relationship were valid, a reliable and cost-effective method could be developed to identify or screen for aircrew susceptibility to DCS. The purpose of this study was to 1) investigate the possibility of using an EIA technique in place of an RIA technique to determine levels of complement activation in response to VGE, and 2) determine if historically VGE-prone subjects have different

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capabilities for complement activation, by bubbles, which is related to their susceptibility to DCS symptoms.

METHODS AND MATERIALS

Subjects

Eight human subjects participated under the Armstrong Laboratory protocol titled "Development of an Enzyme Immunoassay for Complement Proteins to Predict DCS-Prone Individuals." The subjects were healthy, free from known infections, had no major changes in their health status since the time of their last exposure, and had passed the appropriate Altitude Test Subject physical. They were HIV negative and otherwise representative of the USAF rated aircrew population. The subjects were briefed that no scuba diving, other hyperbaric exposures, hypobaric exposures, or flying would be permitted for one week prior to the blood draws.

Table 1. Subject Reaction History

SUBJ. #	PROTOCOL ¹	SUBJECT GROUPING ²	REACTION HISTORY ³				TOTAL EXPOSURES
			V,D	V,ND	NV,ND	NV,D	
1	A	Susceptible	2	0	2	0	4
2	B	Susceptible	6	0	2	0	8
3	A,B	Resistant	3	8	1	0	12
4	B	Resistant	2	6	0	0	8
5	A	Resistant	0	4	0	0	4
6	B	Resistant	1	6	1	0	8
7	C	Susceptible	9	0	0	0	9
8	C	Susceptible	4	0	0	0	4
TOTALS			27	24	6	0	57

¹ Protocols: A = 5.46 psia for 4-6 h
 B = 4.46 psia for 4 h
 C = 6.08, 5.46, 4.89, and/or 4.37 psia for 2 h

² Subject grouping:

Resistant Predominant reaction was VGE without DCS.
 ≤ 25% with [VGE, DCS] or [No VGE, No DCS]
 Susceptible Predominant reaction was VGE with DCS.
 [VGE and DCS] and [No VGE and No DCS]

³ V = VGE; NV = No VGE; D = DCS; ND = No DCS; Number of exposures in which the subject reacted as shown

Subjects were selected for this study based on their VGE- and DCS-susceptibility in previous altitude studies. By using subjects who had consistent responses to reduced pressure, we hoped to be able to determine if there is a relationship between DCS susceptibility and levels of activation of selected complement pathway proteins. A review of exposure history and availability of the several hundred subjects exposed from 1983 through 1990 revealed that fewer than 20 had more than three exposures to above 20,000 ft and had consistent reactions to the exposures. Only eight of those subjects were still available for sampling. The two consistent-reacting groups shown in the tables are described in footnote #2 to Table 1 as "Resistant" and "Susceptible."

Venipuncture and Sample Treatment

Each subject was scheduled to have blood drawn on days 2, 8, and 29 after the initial blood draw. Blood was drawn into 30-ml polypropylene syringes with 20-gauge needles. The blood was transferred via 15-cm polyethylene transfer pipets to polypropylene tubes containing a final concentration of 2 mM Na₂-EDTA. Ward's procedure (17,18,21) specifies use of Na heparin, 7-10 IU/ml, because it gave more consistent results than EDTA in the triplicate samples (14). Logue (8) reported that at the level used by Ward, heparin would inhibit C3 activation depending on other experimental conditions. On the basis of Logue's report (8), use of EDTA during complement studies by other researchers (3,5,10,12), and information from technical support personnel at Amersham and Quidel that heparin could interfere with the analysis of complement activation, 2mM disodium EDTA was used as the anticoagulant (7) at approximately 40% of the strength used by Satoh et al. (10).

The equal aliquots of blood were centrifuged at 3500 rpm (TRIA Centrifuge Model 0200) for 10 min at room temperature. Using disposable polyethylene transfer pipets, 5-7 ml aliquots of plasma were transferred to 15-ml polypropylene tubes and incubated for 30 min at 37°C. One aliquot was incubated with bubbles and the control was incubated without bubbles.

Bubbles entered the plasma from 1-ml polypropylene automatic pipette tips using a compressed air source metered through Nupro fine-metering needle valves at a rate of approximately 60-75 bubbles per minute. This method avoids the "vigorous" shaking and agitation prior to incubation as described by Ward et al. (18,22). Greater rates tended to cause excessive foam above the sample. The diameter of the bubbles was determined by comparison with the polypropylene automatic pipette tips. The 3-4 mm bubbles yielded a total bubble surface area exposed to the plasma of 0.05-0.1 m² ($\pi d^2 \times (\text{bubbles/min}) \times 30 \times 10^{-6}$). The surface area of the plasma exposed to air at the surface of the sample was 1.8X10⁻⁶m² during the incubation of all samples. These bubbles are much larger than the 100 μ m *in-vivo* bubbles known as VGE.

Spontaneous activation of complement occurs during incubation at 37°C. Analogous spontaneous activation in the control and test samples allows determination of the activation due to bubbling. Stabilizing solution was not used for the SC5b-9 EIA (as directed by Dr. Kolb) (7). The incubated plasma

samples were frozen at -70°C to allow batch analyses when all samples had been gathered. One group of incubated plasma samples was frozen for six weeks and analyzed with available EIA and RIA kits. Three sets (control and test) of these samples were refrozen and rethawed with the remaining plasma samples six months after collection and analyzed for Bb and iC3b.

EIA and RIA Analyses

When the frozen plasma samples were thawed, duplicate analyses were accomplished on each sample. The EIA analyses for Bb, iC3b, and SC5b-9 were accomplished using kits from Quidel Inc. Dilution and sample handling were coordinated with Quidel. All assays were done at Wilford Hall Medical Center Clinical Investigation Directorate. Samples were diluted for the EIA analyses (7) as follows: Bb, 1:10; SC5b-9, 1:5; iC3b, 1:25. RIA analyses were accomplished using C3a Arg [^{125}I] and C5a des Arg [^{125}I] RIA kits from Amersham International of Amersham, UK. Only part of the samples were analyzed by both EIA and RIA methodologies due to timing of RIA and EIA kit availability. RIA analyses were accomplished on plasma from the two subjects susceptible to DCS only when VGE were detected (Subjects #1 & 2) and from the two most resistant subjects (Subjects #5 & 6).

The Bb (EIA) analyses quantify specific complement alternative pathway activation. The SC5b-9 (EIA) and C5a (RIA) analyses quantify terminal pathway complement activation resulting from activation of either the classical or alternate pathway. The iC3b (EIA) and C3a (RIA) analyses quantify proteins common to both the classical and alternate pathways of complement activation. Comparing results from these analyses could help identify the most reliable single analysis for determining activation of complement by VGE. A two- to five-fold increase in iC3b was observed in the samples which had been thawed twice. Results from the second analyses were therefore not included in the tables or statistical analyses. The additional activation was apparently due to the first thaw and should be considered in future sample-handling procedures.

Zymosan, derived from yeast, is a potent activator of some complement proteins, in particular, the alternate pathway proteins. To compare the current data with previously reported responses to zymosan (13,18), aliquots of representative control and bubbled samples were thawed and incubated for 30 min with zymosan prior to analysis for C3a, C5a, iC3b, and Bb using the procedure developed by Wagner and Hugli (13). Zymosan should activate all of the proteins tested by the kits used in this study.

RESULTS

Results from enzyme-immunoassay (EIA; Bb, iC3b, and SC5b-9) and radio-immunoassay (RIA; C3a, C5a) of complement proteins are shown in Table 2A for DCS-resistant subjects and Table 2B for DCS-susceptible subjects. Tables 2A and 2B are summarized in Table 3.

Although the mean level of SC5b-9 in incubated-control samples was within the range for normal human plasma (see Table 3), the levels of SC5b-9 in both

incubated-control and incubated-bubbled samples showed extreme intra-subject variability. Therefore, even though the samples incubated with bubbles showed 67% more activation of SC5b-9 than samples incubated without bubbles, mean values for the groups were not significantly different. Intra-subject variability also prevented statistical significance between the higher level of SC5b-9 activation in samples from the susceptible group and SC5b-9 activation in the resistant subjects.

C3a, C5a, and iC3b showed significant activation by bubbles. C3a showed a significant increase (Table 3) in both control and bubbled samples due to inclusion of zymosan. C5a did not show expected activation by zymosan.

Table 2A. Complement Activation in Resistant¹ Subjects

SUBJECT# SAMPLE#	SAMPLE TREATMENT	Quidel Bb µg/ml	Quidel BbZym ² µg/ml	Amersham C3a ng/ml	Amersham C3aZym ² ng/ml	Quidel iC3b µg/ml	Quidel iC3bZym ² µg/ml	Quidel SC5b-9 ng/ml	Amersham C5a ng/ml	Amersham C5aZym ² ng/ml
5 11.3	Control Bubbled	1.07 1.02		259 356	651 1473	13.0 11.2	7.9 11.2	345 677	14.8 12.8	11.2 12.8
5 12.2	Control Bubbled	1.08 1.08				8.9 16.8				
5 13.3	Control Bubbled	1.07 1.16		276 332	735 952	5.7 11.6		74 94	10.8 17.6	9.2 12.0
#5 AVG	Control Bubbled	1.07 1.09		268 344	693 1213	9.2 13.2	7.9 11.2	210 386	12.8 15.2	10.2 12.2
6 11.5	Control Bubbled	1.04 1.06		443 436	799 1420	13.8 18.4		29 76	13.2 14.4	11.2 10.4
6 12.4	Control Bubbled	1.02 1.95				24.1 25.1				
6 13.4	Control Bubbled	0.96 0.97		264 494	548 899	12.3 23.1		170 96		
#6 AVG	Control Bubbled	1.02 0.99		354 465	674 1160	16.7 22.2		100 86	13.2 14.4	11.2 10.4
3 2.0	Control Bubbled	0.88 0.98				13.4 21.2				
4 12.1	Control Bubbled	1.08 1.00								
4 13.1	Control Bubbled	1.01 0.98								
#4 AVG	Control Bubbled	1.05 0.99								

¹ Resistant as defined in footnote 2 to Table 1; average of duplicate analyses

² Samples incubated with zymosan prior to analysis

Table 2B. Complement Activation in Susceptible¹ Subjects

SUBJECT# SAMPLE#	SAMPLE TREATMENT	Quidel Bb µg/ml	Quidel BbZym ² µg/ml	Amersham C3a ng/ml	Amersham C3aZym ² ng/ml	Quidel iC3b µg/ml	Quidel iC3bZym ² µg/ml	Quidel SC5b-9 ng/ml	Amersham C5a ng/ml	Amersham C5aZym ² ng/ml
1 7.1	Control Bubbled	1.06 1.09		247 363	624 1366	21.0 25.1		482 964	8.4 12.0	11.2 14.0
1 8.2	Control Bubbled					19.7 23.1	17.1 25.6			
1 9.1	Control Bubbled	1.13 1.10		270 313	480 1093	8.9 10.2		85 413	12.8 11.6	11.6 17.2
#1 AVG	Control Bubbled	1.10 1.10		259 338	552 1230	16.5 19.7	17.1 25.6	284 688	10.6 11.8	11.4 15.6
2 9.2	Control Bubbled	1.14 1.13		256 391	734 881	10.8 12.8	41.5 57.0	217 267	10.4 12.4	12.0 9.6
2 10.1	Control Bubbled	1.09 1.09				10.3 22.7	31.2 34.1			
2 11.2	Control Bubbled	1.06 1.03	1.03 0.97	284 404	591 722	8.1 11.9		413 439	12.0 16.0	12.4 14.8
#2 AVG	Control Bubbled	1.10 1.08	1.03 0.97	270 398	663 802	9.7 15.8	36.4 45.6	315 353	11.2 14.2	12.2 12.2
7 3.0	Control Bubbled					13.0 18.6				
7 4.0	Control Bubbled					14.5 18.2				
7 5.0	Control Bubbled					13.2 16.5				
7 8.1	Control Bubbled	0.91 0.90				13.5 13.5				
#7 AVG	Control Bubbled	0.91 0.90				13.6 16.7				
8 3.1	Control Bubbled	1.22 1.16				14.8 27.4				
8 4.1	Control Bubbled					23.9 27.0				
8 7.2	Control Bubbled	0.89 0.94				13.6 15.2				
#8 AVG	Control Bubbled	1.06 1.05				17.4 23.2				

¹ Susceptible as defined in footnote 2 to Table 1; average of duplicate analyses² Samples incubated with zymosan prior to analysis

Table 3. Summary of Results

SUBJECT REACTION	SAMPLE TREATMENT	Alternate pathway			Either pathway			Terminal pathway		
		Quidel Bb µg/ml	Quidel BbZym ¹ µg/ml	Amersham C3a ng/ml	Amersham C3aZym ¹ ng/ml	Quidel iC3b µg/ml	Quidel iC3bZym ¹ µg/ml	Quidel SC5b-9 ng/ml	Amersham C5a ng/ml	Amersham C5aZym ¹ ng/ml
Resistant ^a	Control	1.00 ± 0.09(4)		310 ± 61(2)	683 ± 14(2)	13.1 ± 4(3)	7.9 (1)	154 ± 78(2)	13.0 ± 0.3(2)	10.7 ± 0.7(2)
	Bubbled	1.01 ± 0.05(4)		404 ± 86(2)	1186 ± 37(2)	18.9 ± 5(2)	11.2 (1)	236 ± 212(2)	14.3 ± 0.6(2)	11.4 ± 1.4(2)
	Difference	0.01		94	503	5.8	3.3	82	1.8	0.7
Susceptible ^a	Control	1.04 ± 0.09(4)	1.03 (1)	264 ± 8(2)	607 ± 78(2)	14.3 ± 3(4)	26.7 ± 14(2)	299 ± 22(2)	10.9 ± 0.4(2)	11.8 ± 0.6(2)
	Bubbled	1.03 ± 0.09(4)	0.97 (1)	368 ± 42(2)	1016 ± 303(2)	18.8 ± 3(4)	35.6 ± 14(2)	521 ± 237(2)	13.0 ± 1.7(2)	13.9 ± 2.4(2)
	Difference	-0.01	-0.06 (1)	104	409	4.5	8.9	222	2.1	2.1
All ^a	Control	1.02 ± 0.08(8)	1.03 (1)	287 ± 44(4)	645 ± 63(4)*	13.8 ± 3(7)	20.4 ± 15(3)	227 ± 96(4)	12.0 ± 1.2(4)	11.2 ± 0.8(4)
	Bubbled	1.02 ± 0.07(8)	0.97 (1)	386 ± 59(4)	1101 ± 202(4)*	18.9 ± 4(7)	27.4 ± 17(3)	378 ± 247(4)	13.9 ± 1.5(4)	12.6 ± 2.2(4)
	Difference	0.00	-0.06 (1)	99**	456	5.1**	7.0**	151	1.9*	1.4
Normal human plasma		27-89		83-221		6.4-10.6		98-288	0-12	
				25-100					0-50	

Note: Mean values from individual subjects were used to determine group means

* from Table 2A; Mean ± SD (N)

† from Table 2B; Mean ± SD (N)

‡ Resistant and Susceptible; Mean ± SD (N)

1 Samples incubated with zymosan prior to analysis

2 Dr. William Kolb (7)

3 Wagner and Hugli (13)

4 Satoh et al. (10)

* Addition of zymosan resulted in activation; p < 0.05

** Bubbling resulted in activation; p < 0.05

DISCUSSION

The relatively high activation of other complement proteins in control samples reported here compared with normal human plasma probably reflects the effect of sample treatment which included a 30-min control incubation at 37°C. Activation of the complement system during the control incubation at 37°C for 30 min was verified by Ward (14).

Mean levels of Bb in our incubated-control samples were about twice the levels obtained during an earlier study of normal human plasma samples (7) (see Table 3). Although zymosan has been shown to activate Bb in normal human serum (Quidel; 1/90 SL1003), we showed no activation of Bb in incubated normal human plasma by zymosan or bubbles, possibly due to yeast source, shorter incubation time (30 min vs 1 h), and/or use of EDTA as an anti-coagulant. In addition to chelating Ca^{++} to prevent clotting, EDTA chelates Mg^{++} which is required for Bb activation. Chelation of Mg^{++} may be why Bb showed no activation by zymosan or bubbling (7). Future efforts should compare use of EDTA and heparin to determine if Bb is merely unreactive to bubbles and zymosan or if a Bb magnesium requirement is responsible for the inactivity.

C3a activation has recently been reported to be inhibited by both EDTA and heparin (9). EDTA also reportedly limits activation of C3 to about 30% of the total activation occurring in the absence of EDTA (6). Our results indicate that iC3b and especially C3a are not as affected by the potential lack of Mg^{++} as shown by their activation by bubbles in the presence of EDTA (Table 3). The molecular mechanism for this independence and its relation to an air-plasma interface is unknown.

Mean levels of iC3b in incubated-control samples were about twice the levels obtained during an earlier study of normal human blood samples (7) (see Table 3). Samples incubated with bubbles showed significant activation of iC3b, 37%, compared to samples incubated without bubbles. Although the susceptible subject group had zymosan activation which was higher than the zymosan activation of the resistant subject group, no significant difference was found.

Mean values of C3a incubated-control samples were about twice the median level reported for normal human blood (10,13) (see Table 3). Samples incubated with bubbles versus those incubated without bubbles showed significant activation, 35%, of C3a. Aliquots of bubbled and control plasma samples exposed to zymosan yielded significant increases in activation of C3a compared with samples not exposed to zymosan. The increase in activation of C3a resulting from zymosan activation in plasma shown here was much lower than the 30-fold activation of C3a in serum reported by Wagner and Hugli (13) who did not report activation by zymosan in plasma. Due to the reported ten-fold increase of C3a activation in serum versus plasma (13), the corresponding zymosan activation in plasma may also be lower and more consistent with results shown in Table 3.

Mean values of C5a incubated-control samples were within the range reported for normal human blood (10,13) (see Table 3). Zymosan did not activate our C5a control samples in contrast to Wagner and Hugli's (13) report of over 10-fold activation of C5a by zymosan in serum. Satoh et al. (10) reported up to five-fold activation of C5a by clotting the blood to form serum and an additional 18-fold activation by treatment with zymosan, albeit with a much longer incubation period.

Activation of complement by introducing a plasma-air interface in the form of air bubbles may be the result of denaturation of some proteins (or hydrolysis of the internal C3 thioester bond) (6). This may be an *in-vitro* effect that is not present during *in-vivo* production and venous transport of bubbles. Ward's technique of generating the plasma-air interface involved putting 1.5 ml of plasma in a 1.65-ml polypropylene tube, whereupon "it is vigorously shaken to introduce bubbles" after capping (22). This technique could initiate changes in the plasma which relate more to the shaking than to exposure to a plasma-air interface. Also, once the bubbles are formed, they remain intact throughout the 30-min incubation. Bubbles in the human vascular system would not usually remain there for more than a minute due to transport to the lung where they are effectively scavenged (1,2,4). Long-term presence of bubbles in plasma may cause activation far in excess of normal physiologic response. Bubbles which remain

in the circulation for 30 min would probably be lodged in capillaries, provoking physiologic responses not necessarily related to complement activity.

CONCLUSIONS

The results show significant activation of iC3b and C3a due to the presence of air bubbles. Consistent iC3b activation by air bubbles indicates that this EIA method may offer an acceptable alternative to the RIA method for analyzing complement activation by decompression-induced VGE. SC5b-9 showed a trend toward activation by air bubbles although not to a significant level. Zymosan significantly activated only C3a (bubbled or control) although some zymosan activation of iC3b was evident.

SC5b-9 determined by EIA was highly inconsistent, even among single-subject samples. Bb (EIA) was not activated by air bubbles or zymosan. Addition of magnesium ion to the plasma prior to incubation could help to clarify whether EDTA chelation of magnesium is a factor in Bb activation. Use of heparin in lieu of EDTA may result in more consistent results despite the instruction to use EDTA contained in the Amersham RIA kits. Clarification of the role of magnesium or the air-plasma interface in activation of each reaction of the pathway requires further study.

Complement protein levels in individuals who did not react to VGE versus levels in those who developed DCS in the presence of VGE were not significantly different. Complement may be involved in the sequence of events leading to DCS symptoms for those individuals susceptible to form VGE. However, results from the small percentage of our subject pool who specifically and consistently reacted to VGE with symptoms do not provide support for a cause and effect relationship between VGE and complement activation. For any useful application of complement-mediated DCS susceptibility to occur, formation of VGE is still a prerequisite. In all cases where the exposure is insufficient to result in VGE, susceptibility to form VGE is an additional and still unpredictable variable. The role of complement in DCS symptomatology may also be masked by other cause and effect relationships between bubbles and DCS either within or outside the vasculature.

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