ICMJE INTERNATIONAL COMMITTEE of MEDICAL JOURNAL EDITORS

ICMJE Form for Disclosure of Potential Conflicts of Interest

Instructions

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4. Intellectual Property.

This section asks about patents and copyrights, whether pending, issued, licensed and/or receiving royalties.

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Section 1. Identifying Information

1. Given Name (First Name): Shruti
2. Surname (Last Name): Chaturvedi
3. Date: 01-February-2018

4. Are you the corresponding author? ☐ Yes ☑ No

Corresponding Author's Name: Robert Brodsky

5. Manuscript Title: Germline mutations in the alternative pathway of complement predispose to HELLP syndrome

6. Manuscript Identifying Number (if you know it): 99128-INS-CMED-RV-3

Section 2. The Work Under Consideration for Publication

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Are there any relevant conflicts of interest? ☐ Yes ☑ No

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Are there any relevant conflicts of interest? ☐ Yes ☑ No

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Dr. Chaturvedi has nothing to disclose.

Evaluation and Feedback

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ICMJE Form for Disclosure of Potential Conflicts of Interest

Section 1. Identifying Information

1. Given Name (First Name): Samuel
2. Surname (Last Name): Merrill
3. Date: 31-January-2018

4. Are you the corresponding author? ☐ Yes ☑ No

Corresponding Author's Name: Robert Brodsky

5. Manuscript Title: Germline mutations in the alternative pathway of complement predispose to HELLP syndrome

6. Manuscript Identifying Number (if you know it): 99128-INS-CMED-RV-3

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Dr. Merrill has nothing to disclose.

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Section 1. Identifying Information

1. Given Name (First Name): Jagar
2. Surname (Last Name): Jasem
3. Date: 01-February-2018

4. Are you the corresponding author? ☐ Yes ☑ No

Corresponding Author's Name: Robert Brodsky

5. Manuscript Title: Germline mutations in the alternative pathway of complement predispose to HELLP syndrome

6. Manuscript Identifying Number (if you know it): 99128-INS-CMED-RV-3

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Dr. Jasem has nothing to disclose.

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Section 1. Identifying Information

1. Given Name (First Name): Igor
2. Surname (Last Name): Makhlin
3. Date: 31-January-2018

4. Are you the corresponding author? ☐ Yes ☑ No

Corresponding Author's Name: Robert Brodsky

5. Manuscript Title: Germline mutations in the alternative pathway of complement predispose to HELLP syndrome

6. Manuscript Identifying Number (if you know it): 99128-INS-CMED-RV-3

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Section 1. Identifying Information

1. Given Name (First Name): John
2. Surname (Last Name): Sperati
3. Date: 01-February-2018

4. Are you the corresponding author? ☐ Yes ☑ No

Corresponding Author's Name: Robert Brodsky

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Are there any relevant conflicts of interest? ☑ Yes ☐ No

If yes, please fill out the appropriate information below.

Name of Entity	Grant?	Personal Fees?	Non-Financial Support?	Other?	Comments
Alexion Pharmaceuticals, Inc	☐	☑	☐	☐	Chair of Data Safety and Monitoring Board for clinical trial
Alexion Pharmaceuticals, Inc	☑	☐	☐	☐	Site Principal Investigator, Global aHUS Registry

Section 4. Intellectual Property -- Patents & Copyrights

Do you have any patents, whether planned, pending or issued, broadly relevant to the work? ☐ Yes ☑ No

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Dr. Sperati reports personal fees from Alexion Pharmaceuticals, Inc, grants from Alexion Pharmaceuticals, Inc, outside the submitted work; .

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4. Intellectual Property.

This section asks about patents and copyrights, whether pending, issued, licensed and/or receiving royalties.

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Licensed: The patent has been licensed to an entity, whether earning royalties or not
Royalties: Funds are coming in to you or your institution due to your patent

ICMJE Form for Disclosure of Potential Conflicts of Interest

Section 1. Identifying Information

1. Given Name (First Name): Andrea
2. Surname (Last Name): Baines
3. Date: 01-February-2018

4. Are you the corresponding author? ☐ Yes ☑ No

Corresponding Author's Name: Robert Brodsky

5. Manuscript Title: Germline mutations in the alternative pathway of complement predispose to HELLP syndrome

6. Manuscript Identifying Number (if you know it): 99128-INS-CMED-RV-3

Section 2. The Work Under Consideration for Publication

Did you or your institution **at any time** receive payment or services from a third party (government, commercial, private foundation, etc.) for any aspect of the submitted work (including but not limited to grants, data monitoring board, study design, manuscript preparation, statistical analysis, etc.)?

Are there any relevant conflicts of interest? ☐ Yes ☑ No

Section 3. Relevant financial activities outside the submitted work.

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Are there any relevant conflicts of interest? ☐ Yes ☑ No

Section 4. Intellectual Property -- Patents & Copyrights

Do you have any patents, whether planned, pending or issued, broadly relevant to the work? ☐ Yes ☑ No

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ICMJE Form for Disclosure of Potential Conflicts of Interest

Section 5. Relationships not covered above

Are there other relationships or activities that readers could perceive to have influenced, or that give the appearance of potentially influencing, what you wrote in the submitted work?

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Section 6. Disclosure Statement

Based on the above disclosures, this form will automatically generate a disclosure statement, which will appear in the box below.

Dr. Baines has nothing to disclose.

Evaluation and Feedback

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ICMJE Form for Disclosure of Potential Conflicts of Interest

Section 1. Identifying Information

1. Given Name (First Name): Arthur
2. Surname (Last Name): Vaught
3. Date: 01-February-2018

4. Are you the corresponding author? ☐ Yes ☑ No

Corresponding Author's Name: Robert Brodsky

5. Manuscript Title: Germline mutations in the alternative pathway of complement predispose to HELLP syndrome

6. Manuscript Identifying Number (if you know it): 99128-INS-CMED-RV-3

Section 2. The Work Under Consideration for Publication

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Are there any relevant conflicts of interest? ☐ Yes ☑ No

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Are there any relevant conflicts of interest? ☐ Yes ☑ No

Section 4. Intellectual Property -- Patents & Copyrights

Do you have any patents, whether planned, pending or issued, broadly relevant to the work? ☐ Yes ☑ No

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ICMJE Form for Disclosure of Potential Conflicts of Interest

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Dr. Vaught has nothing to disclose.

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ICMJE Form for Disclosure of Potential Conflicts of Interest

Section 1. Identifying Information

1. Given Name (First Name): Evan
2. Surname (Last Name): Braunstein
3. Date: 31-January-2018

4. Are you the corresponding author? ☐ Yes ☑ No

Corresponding Author's Name: Robert Brodsky

5. Manuscript Title: Germline mutations in the alternative pathway of complement predispose to HELLP syndrome

6. Manuscript Identifying Number (if you know it): 99128-INS-CMED-RV-3

Section 2. The Work Under Consideration for Publication

Did you or your institution **at any time** receive payment or services from a third party (government, commercial, private foundation, etc.) for any aspect of the submitted work (including but not limited to grants, data monitoring board, study design, manuscript preparation, statistical analysis, etc.)?

Are there any relevant conflicts of interest? ☐ Yes ☑ No

Section 3. Relevant financial activities outside the submitted work.

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Are there any relevant conflicts of interest? ☐ Yes ☑ No

Section 4. Intellectual Property -- Patents & Copyrights

Do you have any patents, whether planned, pending or issued, broadly relevant to the work? ☐ Yes ☑ No

ICMJE Form for Disclosure of Potential Conflicts of Interest

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Section 6. Disclosure Statement

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Dr. Braunstein has nothing to disclose.

Evaluation and Feedback

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Royalties: Funds are coming in to you or your institution due to your patent

ICMJE Form for Disclosure of Potential Conflicts of Interest

Section 1. Identifying Information

1. Given Name (First Name): Karin
2. Surname (Last Name): Blakemore
3. Date: 01-February-2018

4. Are you the corresponding author? ☐ Yes ☑ No

Corresponding Author's Name: Robert Brodsky

5. Manuscript Title: Germline mutations in the alternative pathway of complement predispose to HELLP syndrome

6. Manuscript Identifying Number (if you know it): 99128-INS-CMED-RV-3

Section 2. The Work Under Consideration for Publication

Did you or your institution **at any time** receive payment or services from a third party (government, commercial, private foundation, etc.) for any aspect of the submitted work (including but not limited to grants, data monitoring board, study design, manuscript preparation, statistical analysis, etc.)?

Are there any relevant conflicts of interest? ☐ Yes ☑ No

Section 3. Relevant financial activities outside the submitted work.

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Are there any relevant conflicts of interest? ☐ Yes ☑ No

Section 4. Intellectual Property -- Patents & Copyrights

Do you have any patents, whether planned, pending or issued, broadly relevant to the work? ☐ Yes ☑ No

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ICMJE Form for Disclosure of Potential Conflicts of Interest

Section 5. Relationships not covered above

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Dr. Blakemore has nothing to disclose.

Evaluation and Feedback

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ICMJE Form for Disclosure of Potential Conflicts of Interest

Section 1. Identifying Information

1. Given Name (First Name): Robert
2. Surname (Last Name): Brodsky
3. Date: 30-January-2018

4. Are you the corresponding author? ☑ Yes ☐ No

5. Manuscript Title: Germline mutations in the alternative pathway of complement predispose to HELLP syndrome

6. Manuscript Identifying Number (if you know it): 99128-INS-CMED-RV-3

Section 2. The Work Under Consideration for Publication

Did you or your institution **at any time** receive payment or services from a third party (government, commercial, private foundation, etc.) for any aspect of the submitted work (including but not limited to grants, data monitoring board, study design, manuscript preparation, statistical analysis, etc.)?

Are there any relevant conflicts of interest? ☑ Yes ☐ No

If yes, please fill out the appropriate information below. If you have more than one entity press the "ADD" button to add a row. Excess rows can be removed by pressing the "X" button.

Name of Institution/Company	Grant?	Personal Fees?	Non-Financial Support?	Other?	Comments
NHLBI	☑	☐	☐	☐	

Section 3. Relevant financial activities outside the submitted work.

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Are there any relevant conflicts of interest? ☑ Yes ☐ No

If yes, please fill out the appropriate information below.

Name of Entity	Grant?	Personal Fees?	Non-Financial Support?	Other?	Comments
Alexion Pharmaceuticals	☑	☐	☐	☑	Advisory Board
Achillion Pharmaceuticals	☐	☐	☐	☑	Advisory Board
Apellis Pharmaceuticals	☐	☐	☐	☑	Advisory Board

ICMJE INTERNATIONAL COMMITTEE of MEDICAL JOURNAL EDITORS

ICMJE Form for Disclosure of Potential Conflicts of Interest

Section 4. Intellectual Property -- Patents & Copyrights

Do you have any patents, whether planned, pending or issued, broadly relevant to the work? ☑ Yes ☐ No

If yes, please fill out the appropriate information below. If you have more than one entity press the "ADD" button to add a row. Excess rows can be removed by pressing the "X" button.

Patent?	Pending?	Issued?	Licensed?	Royalties?	Licensee?	Comments	
modified ham test	☑	☐	☐	☐			

Section 5. Relationships not covered above

Are there other relationships or activities that readers could perceive to have influenced, or that give the appearance of potentially influencing, what you wrote in the submitted work?

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Dr. Brodsky reports grants from NHLBI, during the conduct of the study; other from Alexion Pharmaceuticals, other from Achillion Pharmaceuticals, other from Apellis Pharmaceuticals, outside the submitted work; .

Evaluation and Feedback

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Definitions.

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Corresponding Author's Name: Robert Brodsky

5. Manuscript Title: Germline mutations in the alternative pathway of complement predispose to HELLP syndrome

6. Manuscript Identifying Number (if you know it): 99128-INS-CMED-RV-3

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Dr. Eloundou has nothing to disclose.

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Corresponding Author's Name: Robert Brodsky

5. Manuscript Title: Germline mutations in the alternative pathway of complement predispose to HELLP syndrome

6. Manuscript Identifying Number (if you know it): 99128-INS-CMED-RV-3

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Itemized responses to reviewer comments:

Reviewer A:

Comment: Pooling HPC and TTP may be useful to increase numbers in the control group, but is suboptimal, somewhat contrived.

Response: Pooling of the HPC and TTP groups was done to create a "TMA during pregnancy" control group for comparison to HELLP syndrome. It was not our intention to misrepresent the data, and we presented the data both with and without pooling in the manuscript text. We have now added this data to Tables 5 and 6, and made it more prominent in the results section of the manuscript (page 7 lines 22-24 and page 8 lines 21-22).

Comment: HELLP vs composite control is the only statistically convincing comparison. "No difference" between HELLP and aHUS cohorts is weak, based on small numbers.

Response: We appreciate the reviewer's critique and concur that a major limitation of this study is the small sample sizes of the patient groups. We now state this in the discussion (page 10 line 24). However, both HELLP and aHUS are rare diseases and our study is the largest prospective analysis of HELLP syndrome to date.

Comment: Restricting the analysis to rare variants is not necessarily the best way to enrich for functionally important variants and seems arbitrary or needlessly blunt. For example, activated protein C resistance (FV Leiden) is quite common, and pathogenic. Some common variants in APC genes must have been studied functionally. Could be interesting to include all variants and analyze based on known or predicted functional consequences, then discuss as appears useful.

Response: We thank the reviewer for this comment and agree that one of the disadvantages of the methodology we used to perform variant calling is the loss of some potentially functional variants. We chose a more stringent analysis of our sequencing data in order to minimize false positive calls (ie non-pathogenic variants) in an unbiased manner, similar to published reports for aHUS (ie Thergaonkar et al., 2017). Further, most known variants have not been studied functionally, or have only been studied with in vitro *assays of unclear clinical relevance such as erythrocytes. A single membrane attack complex is sufficient to lyse erythrocytes; nucleated cells (e.g, endothelial cells) require multiple membrane attack complexes to kill the cell. In future studies with larger patient groups, it may be possible to apply a less stringent methodology as suggested.*

Comment: claiming a PPV of 100% is not convincing. With such small numbers, the confidence intervals around the "100%" value must be large, but were not mentioned.

Response: We thank the reviewer for this comment and agree that that the small sample sizes in our study make these predictions less reliable. We have removed these statistical calculations from the manuscript and now report only the data in the discussion (page 10, lines 11-16).

Reviewer B:

Comment: While the authors state that complement levels increase in pregnancy, what information is available concerning complement activation in normal pregnancy? And what is known about the effects of normal pregnancy on levels of complement regulatory proteins?

Germline mutations in the alternative pathway of complement predispose to HELLP syndrome

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Conflict of interest statement: RAB is currently has served as an Alexion Pharmaceuticals Scientific Advisory Board member, an Achillion Pharmaceuticals Scientific Advisor (or consultant), and an Apellis Pharmaceuticals Scientific Advisor (or consultant).

Response: We have specified the complement proteins that increase during normal pregnancy in the discussion (page 11 lines 10-15), and have added an additional reference of primary data. Thus, we now include 5 references to support this statement.

Comment: For readers unfamiliar with HELLP and partial HELLP criteria, a table listing these would be useful in understanding the MS.

Response: We agree and have added a table with HELLP criteria to the supplemental data (Table S1).

Comment: Were any of the placentas of HELLP patients examined after terminations or post-partum? What is known concerning the expression of complement regulatory proteins in the placental trophoblast or other placental cells? IF assessed in this study, was there evidence of increased complement degradation products in these placentas? Does this correlate with mHAM testing.

Response: All placentas were sent to pathology for clinical examination for diagnosis HELLP syndrome. Gross pathology revealed unremarkable placentas and non-specific findings, i.e., inflammation, thombohematomas which can be associated with both labor and manual placental extraction from the uterus. No immunohistochemistry for complement was performed on these specimens so we unfortunately are not able to assess whether or not it correlated with mHam testing. We agree that this would be interesting to investigate going forward.

Comment: Did HELLP resolve after delivery in the patients who had terminations? This is generally thought to occur but is not always the case. Does positivity in the mHAM predict response to termination?

Response: HELLP resolved in all patients post-partum, consistent with our hypothesis that pregnancy is an important trigger for disease onset. This information has been added to the results (page 7 lines 6-8). We are unable to conclude whether mHam positivity predicts response to termination.

Comment: Does the low incidence of mHAM positivity in partial HELLP suggest a different pathophysiology than HELLP per se?

Response: Based on our data of a high prevalence of mHAM positivity and/or a rare germline variant in a gene that regulates the APC in HELLP patients, we feel that a large percentage of HELLP is driven by over-activation of the APC. However, we remain open to the likelihood that not all HELLP is driven by complement. It is even more likely that there are alternative causes of partial HELLP. Certainly there are cases of "aHUS" that don't respond to eculizumab or are mHam negative, also implying other pathophysiologies in some cases of aHUS. We have now commented on this in the discussion (page 10, lines 25-6, page 11 lines 1-3).

Comment: What is the incidence of positive mHAM in patients with CFHR1/CHFR3 deletion-would H5 not be expected to be positive?

Response: There are 2 mHam positive patients with only this variant in the aHUS cohort. However, CFHR1/CHFR3 deletion alone does not appear to produce mHam positivity (as evidenced by multiple pHELLP samples with this variant and a negative mHam). The reason for mHam negativity in case H5 is

unclear; clarification has been added to the results (page 9, lines 19-21).

Comment: Some increased background information on the reason for terminations would be useful. To my knowledge, SGA per se is usually not an indication. Or were there other maternal factors that led to termination--and most importantly did they all improve afterwards?

Response: The reviewer is correct, SGA alone is not an indication for termination of pregnancy. However, in the setting of worsening of the maternal condition secondary to HELLP syndrome along with a fetus less than 500 grams, current recommendations are to offer termination of pregnancy. The fetus, even if above 23-24 weeks gestation, would be considered non-viable in the setting of fetal growth restriction or fetal weight less than 500 grams. In all cases, reasons for termination are provided in the manuscript, and no other factors were involved. This has been clarified in methods section (page 16, lines 14-16).

Comment: It is interesting that the positive mHAMs in some of the HPCs were attributed to increased complement levels in normal pregnancy. To what degree must this occur to cause a positive mHAM?

Response: The mHam is a functional assay that measures that ability of a nucleated cell to regulate complement. Similar to a thrombin time, the test is qualitative and not quantitative. For example, patients with 60% killing don't necessarily have more severe disease than patients with 25% killing (page 7, lines 19-22). Our previous studies have shown that virtually all healthy control subjects are negative in the mHam (< 20.5%). As stated in references 9 and 27, complement activation increases in the second and third trimesters of pregnancy; thus, it is not surprising to us that some healthy pregnant females have a false positive mHam. Moreover, we have previously demonstrated in aHUS that some patients remain positive in the mHam even after achieving remission and discontinuing eculizumab. This clearly fits with a two-hit hypothesis for aHUS (predisposing mutations leading to increased complement activation combined with a strong complement trigger to produce the onset of disease). Thus, a positive mHam by itself does not define aHUS or HELLP. Rather, the mHam is positive in most cases of aHUS and HELLP. These may be the best patients to be considered for future clinical trials of complement inhibition.

Reviewer C:

Comment: The authors have already published a similar paper (Exp. Hem. 2016-Ref 13) in which they describe roughly similar number of patients – probably the same cohort plus a few additional cases. Please discuss.

Response: We appreciate the reviewers concern regarding our patient cohorts. This was a prospective study, and no patients enrolled were previously analyzed. The dates of participant recruitment are now stated in the results and methods to address this reasonable concern (page 14, line 3, 7-8, 10-11).

Comment: The new data are that the investigators performed TDS on 9 genes in these three patient populations (HELLP, aHUS, partial HELLP) and in a control group. Thus, I presume that the clinical descriptions are of the same patient populations as previously published? In any case, the numbers unfortunately remain small. The sample size appears to be is inadequate to make solid conclusions.

Response: Similar to reviewer A, we appreciate the limitations of our study and have added this to the discussion (page 10, line 24). Clinical descriptions of all patient groups are provided in Tables 1-4. The HELLP patients described are new patients as described above.

Comment: Another problem is the so-called "mHam" assay. For example, about 50% of the HELLP

patients had a normal mHam test. Moreover, two controls had very high mHam for which no explanation is provided – just speculation. This should be investigated.
Comment: How do the authors explain that only 5/11 HELLP pts and 3/14 partial HELLP pts but 2/17 control pts have a positive mHam test? Further, HPC group has 42% and about 90% cell killing. This makes no sense whatsoever.

Response: We appreciate the reviewer's critique and have reinforced in the manuscript that HELLP syndrome may have other etiologies (see response above and page 10 lines 24-26, page 11 lines 1-3). We found that 62% of HELLP patients (and ~20% of partial HELLP patients) had a positive mHam test, supporting the hypothesis that complement is highly activated and a potential driver of disease in most cases of classical HELLP; the etiology of partial HELLP is likely more heterogeneous. The numbers above quoted by the reviewer appear to refer to the incidence of rare variants (not mHam positivity). We find that germline variants in APC genes are present in HELLP patients at a similar incidence to that of patients with aHUS. Remember, only 40-50% of aHUS patients harbor rare variants in APC related genes yet the overwhelming majority are mHam positive and respond to terminal complement inhibition.

Comment: The authors also show that ~50% of HELLP patients have a complement AP defect analogous to those observed in aHUS. However, again only a fraction of these patients have an abnormal mHam test. It is a problematic test for several reasons (see below). Critically, in the current series we are not provided with antigenic levels of the plasma proteins or flow data on the membrane proteins. We are missing these data. The workup should consist of quantitative levels (Is haploinsufficiency present?) If not, then functional assessments are necessary if the antigenic levels are normal. Missing data. The authors should prepare the mutant proteins in question, determine if they are expressed by their cell line, and, if so, then perform C3b binding and cofactor activity analyses.

As noted above, the mHam is a problem (Fig 1). The cutoff is 20.5%. About 50% of the HELLP patients fall in the normal range. Further, the mean, for example, of the HELLP patients is ~22%. Thus, mHam as currently structured is just not robust enough for the job, as is evident by visual inspection of Fig 1. In sum, the separation of HELLP from controls is such that it is inadequate to make conclusive statements about a variant. It needs to be correlated with protein measurements, and, most importantly, expression of the purified protein combined with more standard functional assays.

Response: The mHam assesses complement activation on the surface of a nucleated cell as a measure of relative cell death. It is a binary assay. The magnitude of cell killing does not directly correlate to the severity of the TMA (see response above). Thus, statistical analysis was performed using the mHam as a binary measure (positive or negative). This is now stated in the results (page 7, lines 19-22). In addition, we have modified Figure 1 in an attempt to display the data in a more clear and transparent fashion. Each data point is shown, with the median and interquartile range given, allowing for the visualization of the subset of HELLP syndrome cases which are mHam positive.

The majority of variants identified in our HELLP and partial HELLP cohorts were nonsense frameshift mutations or homozygous deletions, which are presumed to result in loss of protein expression. While the SNVs identified in our study could potentially be tested using standard functional assays, these generally make use of non-nucleated cells (ie sheep red blood cells) to test for complement activation. We agree with the reviewer that a major challenge in this field is assigning function to variants (common and uncommon). This is especially difficult for soluble factors such as CFH, C3 and CFHR. Red cell assays have been used to assign function, but are problematic. A single membrane attack complex landing on a red cell is sufficient to induce cell lysis. A major reason for developing the mHam is that we and others have found erythrocytes to be too sensitive for assigning functional significance. The

target organs in aHUS, HELLP, TTP, etc are chiefly nucleated endothelial cells. It takes scores of membrane attack complexes to permanently damage these cells. We originally developed the mHam using endothelial cells but showed that the TF1 line was a good surrogate and much easier to work with since it is a non-adherent cell-line (ref 20).

Thus, protein levels and functional assays, including the mHam, are imperfect. By using an assay the assesses complement resilience of nucleated cells and limiting variant calls to those present in less than 1% of the population, we feel we are likely underestimating the percentage of HELLP patients whose disease is driven by the APC. Nevertheless, applying these stringent criteria to healthy controls and/or disease related controls (TTP) we were able to show that the majority of patients with HELLP have levels of complement activation that turns the mHam positive and that rare variants of the APC are over-represented in this population compared to controls. The ultimate proof for this hypothesis will be to do a randomized controlled trial of complement inhibition in HELLP (beyond the scope of this paper); however, our manuscript provides the framework for performing such a study in the future.

Comment: Also, these data are in contrast to a larger HELLP series (33 patients) that were sequenced and only ~10% had a rare genetic variant – two in FI and one in MCP. (This paper is not noted by the authors – Cravetto, et al. "The genetics of the AP of complement in the pathogenesis of HELLP Syndrome". The Journal of Maternal-fetal Medicine 2012:25, 2322-28). Also, the authors do not reference one of the early reports connecting complement system to HELLP (Blood 2008, Fang et al). Of course, the mHam test does not test for CD46 variants. A larger number of patients needs to be evaluated and by more standard and accepted functional tests of FH, FI and CD46.

Response: We agree with the reviewer that these are important contributions to the literature. A major difference between our study and that of Cravetto et al. is that our study was prospective while this French study was retrospective where virtually all the participants were white. Moreover, Cravetto et al. included a number of participants with SLE and APLS. The discrepancy in incidence of genetic variants may also be explained by differences in methodology. Cravetto et al. used Sanger sequencing to identify variants in 5 genes (compared to 9 in our study). Using these methods, we would have identified germline variants in 1 HELLP patient (8%) and 2 pHELLP patients (14%). This work is now referenced in the discussion (ref 18).

Abstract

Background: HELLP syndrome is a severe variant of hypertensive disorders of pregnancy affecting approximately 1% of all pregnancies, and has significant maternal and fetal morbidity. Previously, we showed that HELLP syndrome has upregulation of the alternative pathway of complement (APC). We hypothesize HELLP syndrome follows a “two-hit” disease model similar to atypical hemolytic uremic syndrome (aHUS), requiring both genetic susceptibility and an environmental risk factor. Our objective was to perform a comparative analysis of the frequency of APC activation and germline mutations in affected women and to create a predictive model for identifying HELLP syndrome.

Methods: Pregnant women with HELLP syndrome, and healthy controls after 20 weeks gestation were recruited, along with aHUS and thrombotic thrombocytopenia purpura participants. We performed a functional assay, the mHam, and targeted genetic sequencing in all groups.

Results: Significantly more participants with rare germline mutations in APC genes were present in the HELLP cohort compared to controls (46% versus 8%, $p = 0.01$). In addition, significantly more HELLP participants were positive for the mHam when compared to controls (62% versus 16%, $p = 0.009$). Testing positive for both a germline mutation and the mHam was highly predictive for the diagnosis of HELLP syndrome.

Conclusion: HELLP syndrome is characterized by both activation of the APC and frequent germline mutations in APC genes. Similar to aHUS, treatment via complement inhibition to mitigate maternal and fetal morbidity and mortality may be possible.

Funding: National Heart Lung and Blood Institute T32HL007525 R01HL133113.

Introduction

Preeclampsia is a devastating multisystem disorder of pregnancy that occurs in 3-5% of all pregnancies, manifesting as hypertension with or without proteinuria and/or end organ damage. Notably, not only does preeclampsia account for maternal morbidity, but it also accounts for 30% of all preterm deliveries, which results in neonatal intensive care unit admissions, increased health care costs, severe neonatal morbidity, and neonatal mortality (1-3). HELLP (hemolysis, elevated liver enzymes, and low platelets) syndrome is the most severe variant of this disorder, and affects approximately 1% of all pregnancies (4). While thought to be due to endothelial cell dysfunction, the precise etiology of both preeclampsia and HELLP syndrome remains unclear, resulting in treatment with supportive regimens such as fetal monitoring, steroids for fetal lung maturity, magnesium for seizure prophylaxis, management of hypertension and ultimately delivery that results in iatrogenic preterm birth (1, 5-7). Furthermore, the diagnostic criteria for HELLP syndrome are ambiguous, with two distinct sets of criteria (Mississippi and Tennessee) based on clinical testing that can be confused by differences in laboratory assays (8).

Complement plays a crucial role in host immunity secondary to the opsonization of pathogens, the recruitment and activation of inflammatory cells, and the initiation of membrane attack complexes (MAC) (9, 10). It consists of an enzymatic cascade of over 30 proteins which are activated by the classical pathway, the lectin pathway, and the alternative pathway (9) . While the classical pathway depends on antigen-antibody complexes (i.e., lupus) for activation, the alternative pathway of complement (APC) is antibody independent and has various triggers including infection, trauma, and pregnancy (11) . In disease states, dysregulation of the APC is frequently secondary to inactivation of regulatory proteins, resulting in amplification loops that increase pro-inflammatory cytokines and activation of C3 and C5, leading to an increase in formation of MAC and subsequent endothelial damage.

Although integral in host survival, the over-activation of complement can cause highly morbid diseases, exemplified by atypical hemolytic uremic syndrome (aHUS). aHUS is caused by APC dysregulation and presents with signs of thrombocytopenia, hemolytic anemia, acute kidney injury, hypertension, and neurologic abnormalities, a clinical presentation phenotypically similar to HELLP syndrome (12, 13). Heterozygous germline mutations in genes that function in the APC are found in approximately 50% of aHUS patients, leading to loss-of-function of complement inhibitors or occasionally gain-of-function in complement activators. Mutations in APC genes have also been identified in small cohorts of patients with preeclampsia and HELLP syndrome (14-21) . While genetic alterations confer susceptibility to disease in aHUS, an additional risk factor, or "trigger", is required for disease onset (12) . Importantly, treatment with terminal complement inhibitors such as eculizumab are effective in treating aHUS (22).

Previously, we assessed activation of the APC in patients with HELLP syndrome using the modified Ham (mHam) test (13). This assay measures the ability of a patient's serum to induce cell death of cells sensitive to complement activity due to a lack of glycosylphosphatidylinositol (GPI) proteins. Without GPI, the cells cannot anchor crucial complement regulator proteins of the APC to their membrane, such as CD55 and CD59, and are unable to regulate complement activation (20). Thus, the mHam is a functional assay that can be used to delineate diseases of APC dysregulation, and has been validated in its ability to distinguish aHUS from thrombotic microangiopathies (TMA) that have similar clinical presentations but differ in pathophysiology (13, 20). We previously reported that like aHUS, a majority of HELLP syndrome patients display dysregulation of the APC in their serum, evidenced by increased cell killing in the mHam assay (13).

Based on our data, we hypothesized that HELLP syndrome follows a "two-hit" disease model similar to aHUS, requiring both a germline susceptibility mutation as well as an environmental risk factor (pregnancy). Here, we show that the APC is more likely to be active in the serum of patients with HELLP syndrome compared to healthy pregnant females. In addition,

we find that patients with HELLP syndrome harbor germline mutations in APC genes at a similar prevalence to that seen in aHUS. We envision this information to be useful in identifying women that have APC dysregulation that may respond to complement inhibition much like other diseases of the APC.

Results

Participant Demographics

To test our hypothesis, women with HELLP syndrome, partial HELLP syndrome, and healthy pregnant controls were recruited from 2015 to 2016. Our cohort consisted of 13 participants with HELLP syndrome (H1-H13, Table 1), 14 participants with partial HELLP syndrome (pH1-pH14, Table 2), and 19 healthy pregnant controls (HPC1-HPC19, Table 3). In order to compare these cohorts to patients with TMA, we also recruited 18 participants diagnosed with aHUS (aHUS1-aHUS18, Table 4), and 8 participants diagnosed with thrombotic thrombocytopenic purpura (TTP1-TTP8, Table 4). The participant demographics among HELLP, partial HELLP, and HPC cohorts did not differ in body mass index (BMI), race, or parity. However, the HELLP group had a statistically significant younger age when compared to the HPC group (27 versus 32 years, $p < 0.030$) but not when compared to partial HELLP syndrome. We also found that the mean estimated gestational age (EGA) of HELLP participants was significantly younger compared to the HPC group (30 versus 37 weeks, $p< 0.001$).

HELLP participants had a significantly greater incidence of adverse maternal and/or fetal events compared to both partial HELLP and HPC groups. Of the HELLP group participants, 7 of 13 (54%) had adverse maternal events. One woman was admitted to the intensive care unit and required renal replacement therapy for acute renal failure. Five women needed blood transfusions, and one participant had an eclamptic seizure. In addition, 7 of 13 women (54%) had adverse fetal events in the HELLP syndrome group. Three participants terminated their pregnancies secondary to the diagnosis of HELLP syndrome in the setting of a non-viable fetus (less than 23 weeks and/or estimated fetal weight less than 500 grams). The other 5 fetuses survived but were diagnosed with fetal growth restriction (FGR) which manifested in the third trimester.

There was one adverse maternal outcome in the partial HELLP group, which was an eclamptic seizure. Further, 4 of 14 participants (29%) experienced the adverse fetal event of

FGR. There were no fetal terminations in the partial HELLP group. In our HPC cohort, there was one participant who was later diagnosed with preeclampsia (not HELLP or partial HELLP syndrome). At the time of recruitment, the participant was normotensive without signs or symptoms of preeclampsia and had an EGA of 38 weeks. She was diagnosed with preeclampsia at 39 weeks and 5 days and this necessitated induction of labor. There was one adverse fetal outcome which was FGR in the HPC group. All HELLP or partial HELLP syndrome resolved post-partum or termination in all cases, and no cases of pregnancy associated aHUS or TTP were observed.

Complement activation in HELLP and aHUS

The mHam assay was performed for all participants with available serum samples in order to assess activation of the APC. A positive test is defined as > 20.5% cell killing, based on our previous study suggesting that HELLP patients are positive in the mHam assay (13). In this analysis, 8 of 13 HELLP participants (62%) were positive, compared to only 2 of 18 participants (11%) in the HPC group (Figure 1). In addition, only 3 of 14 (21%) partial HELLP participants were positive in the mHam assay. For participants with aHUS, 15 of 17 (88%) were positive, while only 1 of 7 participants (14%) with TTP were positive, confirming as expected that the mHam is a useful discriminator between these two phenotypes (20).

While the mHam assesses complement activation as a measure of relative cell death, the magnitude of a result above the 20.5% threshold has unclear significance and has not been found to be associated with clinical outcomes. Thus, statistical analysis was performed using the mHam as a binary measure (positive or negative). When compared to the HPC group, HELLP syndrome participants were significantly more likely to test positive for the mHam assay (62% versus 11%, $p = 0.006$) (Table 5). We also generated a control cohort for a TMA during pregnancy by combining the HPC and TTP groups, as both are expected to have normal complement regulation, and the TTP participants are a confirmed negative control in our

previous study (20). HELLP participants were significantly more likely to test positive in the mHam assay (62% versus 16%, p = 0.009) compared to the composite control cohort. This was also true for aHUS patients compared to this cohort (88% versus 16%, p < 0.001). Conversely, mHam positivity in the HELLP syndrome and aHUS groups was not significantly different. These data indicate that HELLP syndrome, similar to aHUS, is marked by activation of complement.

Identification of rare germline variants in HELLP syndrome

Approximately 50% of aHUS patients harbor germline mutations in genes that regulate the APC; thus, we performed targeted sequencing of participant DNA to identify germline variants in 9 genes known to have a functional role in the APC. To minimize the contribution of likely benign germline polymorphisms, only rare heterozygous germline variants with a minor allele frequency less than 0.01 (1%) were included in our analysis (detailed in methods). In addition, homozygous deletion of *CFHR1* and *CFHR3*, reported to occur in approximately 2% of the population, was included in our analysis due to its association with aHUS and likely functional significance (21). In our pregnant cohorts, 2 participants in the HELLP syndrome group and 2 participants in the HPC group did not give consent for gene mutation testing. We identified a rare germline variant or homozygous deletion in 5 of 11 HELLP participants (46%), compared to only 3 of 14 partial HELLP participants (21%), and 2 of 17 participants (12%) in the HPC group (Tables 1-3, Figure 2). Further, in our TMA cohorts, germline variants were found in 10 of 18 participants with aHUS (56%), while no variants were identified any of the 8 TTP participants (Table 4). When compared to the HPC group alone, there was a non-significant trend towards the presence of a genetic variant in the HELLP syndrome cohort (46% versus 12%, p = 0.07). When compared to the composite controls (combined HPC and TTP groups), HELLP patients were significantly more likely to have a rare germline variant in an APC gene (46% versus 8%, p = 0.01). In addition, there was no difference between the HELLP syndrome and aHUS cohorts for the presence of a genetic variant (Table 6).

Detailed characteristics of all identified genetic variants, along with pathogenicity prediction outcomes for single nucleotide variants via three different algorithms, are provided in Table 7. There were 2 HELLP participants with frameshift mutations, assumed to be loss of function, and 1 participant with a missense mutation predicted to be pathogenic in 2 out of 3 algorithms. Each of these participants also had a positive mHam test. Another HELLP participant with a positive mHam test harbored a homozygous deletion of the *CFHR1* gene. There was one HELLP participant with a negative mHam assay and a germline variant. This individual (H5) was found to have homozygous deletion of both *CFHR1* and *CFHR3* and carried a history of HELLP syndrome in a prior pregnancy. Interestingly, homozygous deletion of *CFHR1* and *CFHR3* was found in 3 participants with partial HELLP, all with negative mHam tests. One of these participants (pH1) also had a history of HELLP syndrome in a prior pregnancy. Of note, pH1 was found to have two additional missense variants in other APC genes as well. There were two HPC participants identified with rare genetic variants, both negative in the mHam assay. One individual (HPC1) carried a diagnosis of sickle cell trait and alpha thalassemia and harbored multiple missense mutations, with one predicted to be pathogenic by all 3 algorithms. By comparison, all aHUS participants with identified germline variants were also found to be positive in the mHam assay. Two participants were found to have deletions of both *CFHR1* and *CFHR3*, while 1 participant harbored a deletion of *CFHR1* only. Deletion of *CFHR* genes were not identified in any HPC or TTP participants, supporting the functional significance of this variant. However, this variant by itself does not appear to sufficiently activate the APC in order to produce a positive mHam test.

Discussion

In this study, we demonstrate that HELLP syndrome is part of the phenome that results from germline mutations in genes that regulate the APC. Using phenotypic, functional (mHam assay demonstrating impaired complement regulation), and genetic (mutations in genes that regulate the APC) data we found striking similarities to aHUS. Our genetic analysis was limited to rare germline variants in order to increase the likelihood of identifying pathogenic mutations that alter the function of APC genes. Using this strict criterion, significantly more APC germline mutations were identified in the HELLP syndrome cohort compared to controls (46% versus 8%, $p = 0.01$), while no difference was found when compared to aHUS. We also confirmed our previous data demonstrating that significantly more HELLP participants test positive in the mHam assay compared to controls (62% versus 16%, $p = 0.009$). Further, we did not identify any individuals in our control groups that tested positive for both the mHam assay and a germline variant (0 of 23 HPC and TTP participants, compared to 4 of 11 HELLP participants). Conversely, both tests were negative in 17 of 23 control participants and only 2 of 11 HELLP participants. Thus, this combination of testing may identify individuals with HELLP syndrome who could potentially benefit from therapeutic intervention.

Our findings agree with previous studies suggesting that complement plays an important role in the pathophysiology of HELLP. The APC activation fragment Bb was found to be a predictor of acquiring preeclampsia (23). In addition, a prospective study of 40 pregnant women with lupus and/or antiphospholipid antibodies who developed preeclampsia identified 7 (17.5%) that had germline heterozygous mutations likely to be deleterious in *MCP*, *CFI* or *CFH* (14). Other studies have performed targeted sequencing of various APC genes in patients with HELLP syndrome and identified heterozygous mutations in 9% to 36% of cases (18, 19).

The major limitation of this study is the small sample sizes of the participant groups. Further, multiple HELLP and partial HELLP participants tested negative for APC activation in the mHam assay. The most likely explanation for this finding is that the pathophysiology of HELLP

syndrome (and partial HELLP even more so) is due to multiple factors. However, this study builds on our previous data and that of others demonstrating that a significant subset of HELLP syndrome is due to a reduced ability to regulate the APC, analogous to aHUS (13, 18, 20). Indeed, the clinical manifestations of hypertension, renal insufficiency, thrombocytopenia, elevated LDH, elevated AST, and even the presence of schistocytes are common to both disorders. Roughly 50% of aHUS patients harbor germline mutations, usually heterozygous, that increase susceptibility of endothelial cells to injury from activated complement (12, 15, 16). These patients are often asymptomatic for decades until they encounter a strong complement "trigger" such as major surgery, infection, malignancy, autoimmunity, or pregnancy. In HELLP syndrome, pregnancy is the likely trigger (11). Previous studies have shown that complement proteins such as CFH and C5b-9, as well as associated contributors of increased complement (i.e. c-reactive protein) are significantly increased during pregnancy, possibly explaining why phenotypic manifestations of HELLP syndrome almost exclusively occur in the late second or third trimester (9, 24-27). This may also explain why two cases in the HPC cohort tested positive in the mHam assay.

In our cohort, HELLP syndrome participants were associated with more frequent adverse maternal and fetal outcomes compared to the partial HELLP syndrome or HPC participants. Women with HELLP syndrome are more likely to have increased morbidity and mortality secondary to DIC and hepatic rupture compared to other hypertensive disorders of pregnancy (28, 29). However, 6 of the 7 women with HELLP syndrome who had maternal adverse events were either positive by the mHam assay or had a rare variant in an APC gene. Our data suggest that the increased morbidity or mortality in HELLP syndrome is associated with dysregulation of the APC. In the partial HELLP group, there was only one participant (pH6) with an adverse maternal outcome (an eclamptic seizure); she was also positive in the mHam indicating increased APC activation. There was one participant in the HPC group with a negative mHam and genetic analysis who was later diagnosed with preeclampsia.

In the HELLP group there were 7 participants who had adverse fetal outcomes, 3 of which were terminations of pregnancy secondary to early gestational age and fetal weight less than 500 grams at time of diagnosis. Two of these had either a positive mHam or a rare germline variant. In the partial HELLP group there were 4 participants with adverse fetal outcomes, all of whom had either a positive mHam test or a rare germline variant, further indicating that abnormal complement regulation is associated with both poor maternal and fetal outcomes. There were 2 HPC participants with positive mHam assays, potentially secondary to the normal upregulation of the complement system during pregnancy, particularly in the third trimester (9). Further, there were 2 HPC participants with rare variants of the APC. Similar to multiple other genetic diseases (hemochromatosis, for example), rare variants of the APC are associated with incomplete penetrance and variable expressivity (30-35). Therefore, genetic variants are best characterized as risk factors, with disease onset occurring in the setting of additional environmental stress causing complement activation.

Our findings have important clinical implications for the diagnosis and treatment of HELLP syndrome. Reliable biomarkers and genetic mutations that predispose to disease states are critical for planning future clinical trials and for targeted approaches to therapy. Paroxysmal nocturnal hemoglobinuria (PNH) and aHUS are diseases caused by mutations that predispose cells to complement-mediated destruction (16, 36-38). Both of these conditions are dramatically and rapidly improved by blocking terminal complement with the humanized monoclonal antibody, eculizumab (22, 39-41). Prior to FDA approval of eculizumab, pregnancy was relatively contraindicated in PNH due to excessive complement activation that increased fetal and maternal mortality. Eculizumab is now routinely recommended for PNH patients who become pregnant since the drug does not effectively cross the placenta or enter the breast milk (42). Complement inhibition has also greatly improved the outcome of patients with aHUS. Recent studies in aHUS show that this treatment can be safely discontinued in most patients once a complete remission is achieved and the complement trigger has resolved (43). Our study

confirms that aHUS and HELLP share similar pathophysiology. Indeed, there is a case report of eculizumab treatment leading to clinical improvement and prolongation of pregnancy in a patient with HELLP syndrome (44). Furthermore, we previously demonstrated that C5 inhibition can effectively block complement-mediated killing in HELLP serum *in vitro* (13). Currently, HELLP syndrome is most effectively managed by delivering the fetus (removal of the complement trigger); however, in cases of early gestational age (before 28 weeks) there is a high risk of fetal mortality and maternal morbidity (1). Based on our data and that of others, we suggest that future trials of complement inhibition in HELLP syndrome include genetic testing and the mHam assay to firmly establish the diagnosis. Patients with HELLP syndrome diagnosed prior to 28 weeks gestational age may benefit from this therapeutic intervention to allow the fetus to reach a safer gestational age and achieve less neonatal morbidity

In summary, most HELLP syndrome and likely some preeclampsia, is driven by failure to regulate the APC. Serologic assays demonstrating a reduced ability to regulate complement (mHam), germline mutations in genes that regulate the APC, or both, are found in a majority of patients with HELLP syndrome. While we do not advocate for off-label use of complement inhibitors in HELLP syndrome, our findings form the basis for the design of clinical trials to test the efficacy of complement inhibition to reduce perinatal morbidity from early prematurity.

Methods

Study population and design

We performed a prospective case-controlled study to identify whether HELLP syndrome is associated with both activation of the APC as well as germline variants in genes that regulate the APC. We hypothesized that HELLP syndrome patients harboring genetic variants of APC genes would also display activation of the APC via a functional assay (the mHam). All participants were recruited from the Johns Hopkins Medical Institute (JHMI), and none had been studied previously. The Tennessee criteria were used for the diagnosis of HELLP syndrome, and partial HELLP syndrome was defined by having one or two but not all lab abnormalities of HELLP syndrome (Table S1) (1, 45). We recruited all participants from November 1, 2015 to December 31, 2016. Healthy pregnant controls were recruited after 23 weeks gestation, and were excluded if they had a history of hypertensive disease, complement or microangiopathic disorder, or autoimmune disease. Blood was collected from all participants at the time of recruitment by venipuncture of the antecubital vein. Serum was cold centrifuged within 2 hours of collection at 4 °C and stored at -80'C. Whole blood was used to generate genomic DNA for targeted gene sequencing using a Qiagen DNeasy blood and tissue kit. All samples were de-identified and coded prior to laboratory testing. Patients with aHUS were recruited using standard diagnostic criteria defined by Legendre et al. 2013 (39). Patients with TTP were recruited following confirmation of the diagnosis via an ADAMTS13 activity less than 10%.

Modified Ham Assay

The modified Ham assay was performed as previously described (20) . Briefly, PNH-like (PIGA –null) TF-1 cells were used to assess complement mediated killing via the APC. Cells were plated in a U-shaped well at a density at 4000 cells and incubated in 20% serum with Gelatin Veronal Buffer (GVB, Complement Technology, Inc). Each sample was tested in triplicate for 30 minutes at 37degrees C. After incubation, the supernatant was removed and

cell proliferation agent WST-1 (Roche) was added and incubated for 2 hours at 37 degrees C. Absorbance was measured in an iMark Microplate Absorbance Reader (Bio-Rad, Hercules, CA) at 490 nm with a reference wave length at 595 nm. Heat inactivated serum was used as a negative control for each sample. Heat inactivation was performed by incubating the serum at 56 degrees C for 30 minutes. Normal human AB serum (H4522, Sigma-Aldrich) was used as an internal control of the assay. The percentage of live cells was calculated as the ratio of the absorbance of each sample divided by the absorbance of the same sample's heat-inactivated control. The percentage of non-viable cells (cell killing) was calculated as 1 minus this ratio.

Targeted sequencing

A custom panel of 9 genes known to function in the alternative pathway of complement was selected for sequencing using the Illumina TruSeq Custom Amplicon (v1.5). Illumina DesignStudio software was utilized to generate custom oligonucleotides designed to amplify exonic regions of the following selected genes: *CFH, CFB, CFI, CFHR1, CFHR3, CFHR5, C3, CD46 (MCP),* and *THBD*. The panel consisted of 402 amplicons covering a total of 58.3 kilobases (kb) with an overall coverage of 98%. An amplicon library of each patient sample was generated according to the manufacturer protocol using 250ng genomic DNA as input. Successful library amplification was verified for a subset of samples by both gel electrophoresis and Bioanalyzer analysis. This was followed by library normalization and pooling per manufacturer protocol, and paired-end sequencing on the Illumina MiSeq platform via the Genetic Resources Core Facility (GRCF) at Johns Hopkins School of Medicine (https://grcf.jhmi.edu). Analysis of raw sequencing data (FASTQ) was performed using the TruSeq Amplicon application (v2.0.0) via the Illumina BaseSpace platform, aligning to (GRCh37/hg19) human genome reference. Average base coverage was 377x per sample. Single nucleotide variants (SNVs) and small indels were identified using Isaac variant caller and variant call files were filtered using VariantStudio software (v3.0). Variants not passing Illumina's

variant quality filters were excluded. The following criteria were used to identify rare germline SNVs and indels: nonsynonymous coding region variants with a depth >20x, variant allele frequency between 40-60%, Exome Aggregation Consortium (ExAC v0.3.1) minor allele frequency < 0.01 in any ethnic population (total of 60,706 samples) and minor allele frequency < 0.01 in the Exome Variant Server (46). Large deletions were determined by complete loss of signal for multiple consecutive amplicons (Figure S1). All genomic coordinates refer to hg19.

Adverse Events

The investigators sought to observe any adverse maternal or fetal events in the HELLP group, partial HELLP group, and HPC group. Maternal adverse events were maternal mortality, intensive care unit admission, renal failure, blood product transfusion, and eclampsia in HELLP or partial HELLP groups, or new diagnosis of preeclampsia in a control group. Fetal adverse events were fetal termination or abortion secondary to diagnosis of HELLP or partial HELLP syndrome, neonatal mortality, and fetal growth restriction. Other than worsening of HELLP syndrome in the setting of a non-viable fetus, no other factors were relevant for fetal terminations. Because neonatal intensive care unit admission in preeclampsia and HELLP syndrome is usually secondary to iatrogenic preterm birth, we did not include this as an adverse event in our group.

Statistics

A one-way ANOVA test with a Bonferroni correction was used to calculate the p-values for the difference among the means for age, BMI, estimated gestational age in HELLP, partial HELLP, and the healthy pregnant control groups. Either Chi-squared test or Fisher's exact test was used to calculate the rest of the p-values provided. A Fisher's exact was used if more than 20% of the expected values in the input cells were less than 5 and/or any individual values had expected counts of less than 1. Statistical significance for the mHam assay was calculated as a

binary value (positive or negative) as the mHam assay is thought to be clinically significant as a qualitative test. All analyses were performed by using SPSS 18 for Windows/MAC (PASW Statistics GradPack 18; SPSS, Chicago, IL). All tests were two-sided with a 0.05 level of significance.

Study approval

This study was approved by the Johns Hopkins institutional review board and conducted according to Declaration of Helsinki principles. Written informed consent was received from all participants prior to inclusion in the study.

Author contributions

AJV and RAB were responsible for the study conception and design. AJV, EMB, RAB, SM, SC, SE, KB and CJS collected and categorized study participants. AJV, EMB, XY, IM and AB performed experiments and acquired data. AJV, EMB and RAB analyzed and interpreted the data. JJ performed statistical data analysis. AJV, EMB, and RAB drafted the manuscript and performed critical revisions. All authors approved the final version of the manuscript.

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Figure Legends

Figure 1: Modified Ham (mHam) results among all participant groups. Each participant is represented as a black circle, and cell killing in the mHAM assay is displayed as a continuous variable for each participant group. The mean and interquartile range of 25-75% is depicted for each group. Statistical analysis demonstrating significant differences between groups using the mHam as a binary measure (positive vs negative) is shown in Table 6. A positive mHam is defined as cell killing of > 20.5% (dashed black horizontal line).

Figure 2: Correlation of germline variants in complement proteins with functional testing. Each genomic variant is identified by its amino acid consequence along with the result of mHam testing in that patient. A filled circle or square represents a positive mHam, while an open circle or square represents a negative test. Patients in which only a single variant was identified are represented with circles, while patients with more than one variant, are represented with squares. Each cohort is designated by a unique color (legend). The total number of amino acids (aa) in each protein are provided. Numbered protein domains denote complement control protein modules (CCP), other protein domains are as labeled. SRCR, scavenger receptor cysteine-rich; LDL, low-density lipoprotein; MG, macroglobulin; ANA, anaphylatoxin; TM, transmembrane;

Table 1: HELLP syndrome participants																
ID	**EGA (wk)**	**Race**	**Age (yr)**	**BMI**	**P**	**SBP / DBP**	**Cr (mg/dL)**	**Highest AST (IU/L)**	**Highest ALT (IU/L)**	**Lowest Platelets (mm^3)**	**Pr / Cr**	**Highest LDH (IU/L)**	**mHAM (%)**	**Germline Variant**	**Adverse Maternal Events**	**Adverse Fetal Events**
H1	31.2	Black	28	34.5	0	169/103	0.7	64	63	85000	0.43	431	32	CFHR1 p.L322Rfs*50	None	FGR
H2	26.4	Black	21	26.9	0	140/80	0.9	321	287	50000	3.20	867	32	C3 p.G542D	None	FGR
H3	34.2	Black	33	28.3	>2	204/121	6.5	520	86	21000	4.50	2030	29	del(CFHR1) homozygous	MV, RRT	None
H4	33	White	35	22.8	0	141/80	0.7	1163	749	28000	1.46	1461	21	CFHR5 p.K562Nfs*24	None	None
H5	24	White	26	30	>1*	150/72	0.7	167	145	100000	0.24	574	8	del(CFHR3-CFHR1) homozygous	None	Termination
H6	17.2	Black	24	23.8	0	165/109	1.2	84	65	77000	0.32	540	33	None	PLT	Termination
H7	38	White	32	47.1	0	180/100	0.9	420	308	43000	0.39	591	30	None	RBC, PLT	None
H8	24.2	Black	22	33.6	0	180/100	1.3	338	170	52000	5.04	2015	23	None	RBC, PLT, Eclampsia	FGR
H9	36	White	23	29.3	0	166/98	0.6	243	181	42000	12.35	581	23	None	PLT	FGR
H10	32.1	Black	15	16.3	0	230/110	0.6	114	81	42000	N/A	612	11	NS	RBC	None
H11	34.2	Black	19	24	0	191/84	0.6	201	101	63000	6.93	325	10	None	None	None
H12	22.2	Black	45	33.7	>2	155/90	0.7	131	125	68000	9.77	503	0	None	None	FGR Termination
H13	37.2	White	27	36.2	0	180/91	0.6	695	423	19000	0.19	805	0	NS	PLT	None

EGA = estimated gestational age, wk = weeks, yr = years, BMI = body mass index, BP = blood pressure, P = parity, SBP (mmHg) = systolic blood pressure, DBP (mmHg) = diastolic blood pressure, Cr = creatinine, AST = aspartate aminotransferase, ALT = alanine aminotransferase, Pr/Cr = protein creatinine ratio, LDH = lactate dehydrogenase, mHam = modified Ham (positive for modified Ham > 20.5%), FGR = fetal growth restriction, MV = mechanical ventilation, RRT = renal replacement therapy, NS = not sequenced, RBC = red blood cell transfusion, PLT = platelet transfusion, * history of prior HELLP syndrome or eclampsia in previous pregnancy.

Table 2: Partial HELLP syndrome participants															
ID	**EGA (wk)**	**Race**	**Age (yr)**	**BMI**	**P**	**SBP / DBP**	**Cr (mg/dL)**	**Highest AST (IU/L)**	**Highest ALT (IU/L)**	**Lowest Platelets (mm3)**	**Pr / Cr**	**Highest LDH (IU/L)**	**mHAM (%)**	**Germline Variant**	**Adverse Maternal Events**	**Adverse Fetal Events**
pH1	32.4	Black	24	25.2	> 1*	169/98	0.6	99	14	176000	0.67	N/A	7	C3 p.P890H, CFI p.I416L, del(CFHR3-CFHR1) homozygous	None	FGR
pH2	30.6	Black	25	32.7	> 2	180/123	2.0	51	45	106000	6.43	492	6	C3 p.D1457H, del(CFHR3-CFHR1) homozygous	None	None
pH3	34	Black	29	35.5	0	176/91	1.0	72	72	164000	12.2	430	4	del(CFHR3-CFHR1) homozygous	None	FGR
pH4	27	White	36	41.7	0	200/80	0.7	78	78	293000	1.52	529	37	None	None	None
pH5	30.4	Asian	26	27.1	0	190/110	0.5	55	66	176000	0.59	184	27	None	None	FGR
pH6	28.1	Black	25	32.4	>1	173/103	0.7	93	90	130000	3.00	554	22	None	Eclampsia	FGR
pH7	37.5	White	41	34	0	157/100	0.8	65	89	140000	0.13	161	20	None	None	None
pH8	37.5	Black	30	42.3	>2	171/90	1.1	792	841	294000	0.45	1124	18	None	None	None
pH9	39.2	Black	34	52.4	0	183/84	0.9	134	74	237000	0.63	284	12	None	None	None
pH10	37.2	Black	29	39.1	0	194/106	0.8	28	23	222000	0.35	434	11	None	None	None
pH11	31.2	White	31	19.6	0	165/71	0.6	22	22	84000	0.86	228	6	None	None	None
pH12	32.6	White	33	28.4	>1	145/77	1.1	181	189	316000	6.23	222	3	None	None	None
pH13	39.3	Black	30	35.5	> 1	180/103	1	76	88	249000	0.20	480	3	None	None	None
pH14	34.4	Asian	27	27.2	0	158/97	0.6	69	91	261000	8.71	317	0	None	None	None

EGA = estimated gestational age, wk = weeks, yr = years, BMI = body mass index, BP = blood pressure, P = parity, SBP (mmHg) = systolic blood pressure, DBP (mmHg) = diastolic blood pressure, Cr = creatinine, AST = aspartate aminotransferase, ALT = alanine aminotransferase, Pr/Cr = protein creatinine ratio, LDH = lactate dehydrogenase, mHam = modified Ham (positive for modified Ham > 20.5%), FGR = fetal growth restriction. * history of prior HELLP syndrome or eclampsia in previous pregnancy.

Table 3: Healthy pregnant control (HPC) participants										
ID	**EGA**	**Race**	**Age**	**BMI**	**P**	**SBP / DBP**	**mHam (%)**	**germline Variant**	**Adverse Maternal Events**	**Adverse Fetal Events**
HPC*	37.3	Black	34	36.6	> 1	128 / 71	8	CFH p.H371N, CFHR5 p.S78P, CFHR5 p.Y279N	None	None
HPC2	38	Black	27	29.8	0	128 / 85	4	CFHR3 p.C15Y	None	None
HPC3	38.1	White	31	26.7	> 1	122 / 76	100	None	None	None
HPC4	40	White	34	26.6	> 1	115 / 74	50	None	None	None
HPC5	39.1	Other	32	37.6	> 3	124 / 83	16	None	None	None
HPC6	39	Asian	36	25.4	> 2	105 / 64	15	None	None	None
HPC7	38	White	35	35.1	0	170 / 100	14	None	Preeclampsia	None
HPC8	38	Black	38	28.6	> 1	121 / 87	13	None	None	None
HPC9	37.1	White	35	22.8	> 1	119 / 67	12	None	None	None
HPC10	36	Black	32	32	> 3	116 / 61	9	None	None	None
HPC11	34.1	White	32	27.5	0	131 / 76	7	None	None	None
HPC12	38.3	Black	20	27.7	> 1	106 / 58	7	None	None	None
HPC13	36.3	Asian	36	30.16	> 1	93 / 53	6	None	None	None
HPC14	37	Black	31	40.8	>3	133 / 62	5	None	None	None
HPC15	39.3	White	37	28.1	0	121 / 84	5	NS	None	None
HPC16	36.2	White	30	27.6	> 2	127 / 76	3	None	None	None
HPC17	38.6	White	33	28.6	0	118 / 75	3	NS	None	None
HPC18	37	White	36	20.5	0	135 / 85	1	None	None	None
HPC19	38	White	28	37.7	>1	110 / 73	N/A	None	None	FGR

* history of sickle cell trait, EGA = estimated gestational age, wk = weeks, yr = years, BMI = body mass index,BP = blood pressure, P =parity, SBP (mmHg) = systolic blood pressure, DBP (mmHg) = diastolic blood pressure, mHam = modified Ham (positive for modified Ham > 20.5%), FGR = fetal growth restriction, NS = not sequenced.

Table 4: aHUS and TTP participants										
ID	**Race**	**Age (yr)**	**Cr (mg/dL)**	**Highest AST (IU/L)**	**Highest ALT (IU/L)**	**Lowest Platelets (mm3)**	**Highest LDH (IU/L)**	**mHAM (%)**	**ADAMTS13 (%)**	**Germline Variant**
TTP1	White	38	0.87	61	37	7	1081	23	<5	None
TTP2	Other	27	1.6	25	23	25	1455	21	<5	None
TTP3	Black	32	2.3	473	187	4	2215	17	<5	None
TTP4	Black	43	1.3	58	41	4	1985	16	<5	None
TTP5	Black	56	0.9	51	20	7	1081	8	<5	None
TTP6	Black	39	1.4	39	22	5	825	5	<5	None
TTP7	Black	60	1.9	30	27	9	2337	5	<10	None
TTP8	Black	64	1.2	53	13	10	1482	n/a	<5	None
aHUS1	Black	47	4.3	28	22	17	1718	63	88	CFB p.I242L, del(CFHR1) homozygous
aHUS2	Black	23	3.7	273	591	8	2024	49	54	del(CFHR1-CFHR3) homozygous
aHUS3	White	32	n/a	n/a	n/a	n/a	n/a	42	n/a	CFHR3 p.G117V
aHUS4	Black	38	6.1	47	20	63	917	39	>100	CFH p.V158I, CFH p.A1027P
aHUS5	White	38	3.48	93	52	45	2501	39	>100	THBD p.A43T
aHUS6	White	32	6.5	26	15	76	495	28	96	CD46 p.W216C
aHUS7	Asian	32	7.2	109	106	7	1588	28	41	CFHR5 p.P453A
aHUS8	White	25	n/a	n/a	n/a	n/a	n/a	27	n/a	CFH p.R1078S
aHUS9	White	78	4	86	32	47	1473	26	53	del(CFHR1-CFHR3) homozygous
aHUS10	White	51	3.7	63	20	18	5266	25	>100	CFH p.R1074P, CD46 p.S274YFS*11
aHUS11	White	22	8.2	68	117	62	2820	49	100	None
aHUS12	White	22	4.3	n/a	n/a	85	471	36	28	None
aHUS13	Black	24	14.9	22	13	77	649	36	55	None
aHUS14	White	50	6.3	55	54	76	903	27	40	None
aHUS15	White	73	1.2	151	150	21	733	24	96	None
aHUS16	White	25	1.4	46	18	20	1160	10	82	None
aHUS17	White	61	1.6	75	85	21	549	9	87	None
aHUS18	White	17	n/a	n/a	n/a	n/a	n/a	n/a	34	None

aHUS = atypical hemolytic uremic syndrome, TTP = thrombotic thrombocytopenia purpura, yr = years, Cr = creatinine, AST = aspartate aminotransferase, ALT = alanine aminotransferase, LDH = lactate dehydrogenase, mHam = modified Ham,

Table 5: Comparison of mHam for all groups						
Participant Group	(+) mHam (%)	(-) mHam (%)	**Participant Group**	(+) mHam (%)	(-) mHam (%)	p-value
HELLP Syndrome	8 (62%)	5 (38%)	**HPC**	2 (11%)	16 (89%)	0.006
HELLP Syndrome	8 (62%)	5 (38%)	**HPC+TTP**	4 (16%)	21 (84%)	0.009
aHUS	15 (88%)	2 (12%)	**HPC+TTP**	4 (16%)	21 (84%)	<0.001
HELLP Syndrome	8 (62%)	5 (38%)	**aHUS**	15 (88%)	2 (12%)	0.19

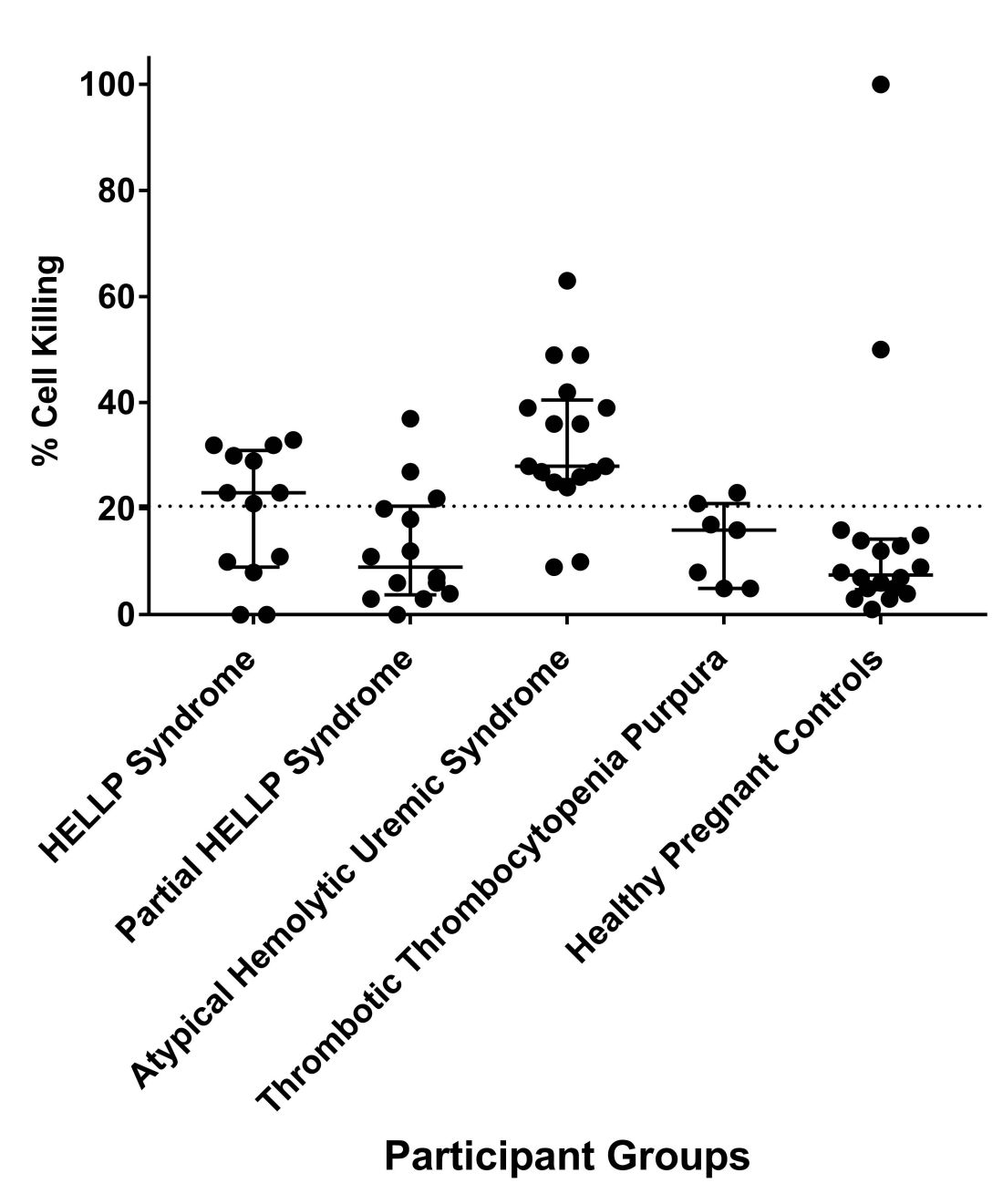
mHam = modified Ham assay, aHUS = atypical hemolytic uremic syndrome, HPC = healthy pregnant control, TTP = thrombotic thrombocytopenia purpura.

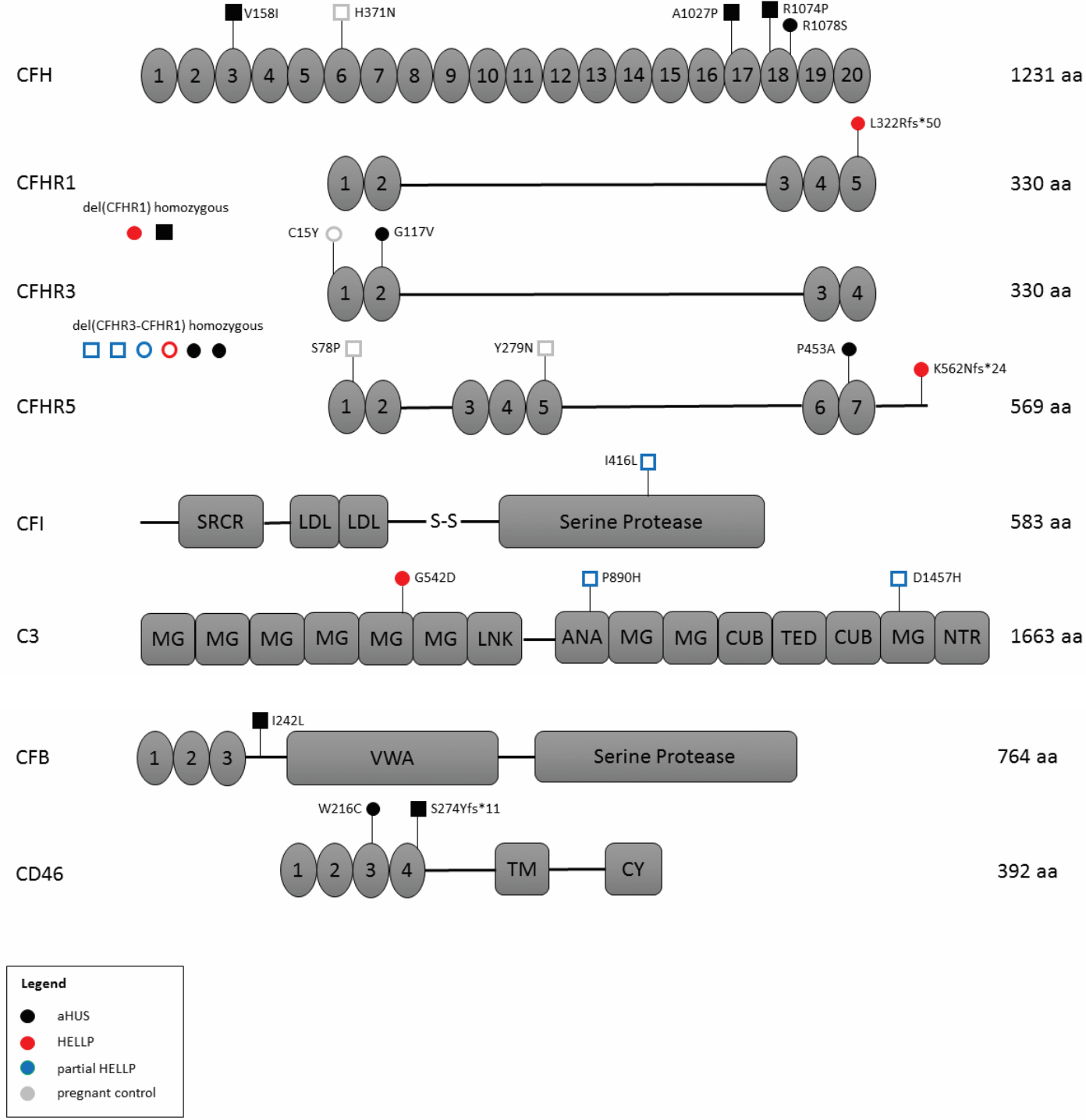
Table 6: Comparison of germline mutation status for all groups						
Participant Group	(+) mutation (%)	(-) mutation (%)	**Participant Group**	(+) mutation (%)	(-) mutation (%)	p-value
HELLP Syndrome	5 (46%)	6 (54%)	**HPC**	2 (12%)	15 (88%)	0.07
HELLP Syndrome	5 (46%)	6 (54%)	**HPC+TTP**	2 (8%)	23 (92%)	0.01
aHUS	10 (56%)	8 (44%)	**HPC+TTP**	2 (8%)	23 (92%)	0.001
HELLP Syndrome	5 (46%)	6 (54%)	**aHUS**	10 (56%)	8 (44%)	0.12

mutation = rare germline variant in a gene of the alternative pathway of complement, aHUS = atypical hemolytic uremic syndrome, HPC = healthy pregnant control, TTP = thrombotic thrombocytopenia purpura.

Table 7: Germline variant characteristics											
ID	**Gene**	**Nucleotide Variant**	**Chr**	**Coordinate**	**VAF**	**Read Depth**	**Protein Position**	**MAF (ExAC)**	**Sift**	**PolyPhen**	**Provean**
H3	C3	C>C/T	19	6710711	56	208	G542D	0.00001682	tolerated	possibly damaging	deleterious
pH1	C3	G>G/T	19	6697482	48	297	P890H	0.00002471	tolerated	benign	deleterious
pH1	CFI	T>T/G	4	110667561	44	424	I416L	0.001113	deleterious	benign	neutral
H4	CFHR5	GGAAATTTGAATATCCTATATGT> GGAAATTTGAATATCCTATATGT/G	1	196977785	40	609	K562Nfs*24	N/A	N/A	N/A	N/A
pH3	C3	C>C/G	19	6680256	44	393	D1457H	0.0003626	deleterious	probably damaging	deleterious
H2	CFHR1	CT>CT/C	1	196801100	59	711	L322Rfs*50	N/A	N/A	N/A	N/A
HPC2	CFHR3	G>G/A	1	196744060	50	277	C15Y	N/A	deleterious	benign	deleterious
HPC1	CFH	C>C/A	1	196658696	49	440	H371N	0.00007426	tolerated	benign	deleterious
HPC1	CFHR5	T>T/C	1	196952188	46	725	S78P	0.0001401	deleterious	probably damaging	deleterious
HPC1	CFHR5	T>T/A	1	196965196	47	554	Y279N	0.0001484	tolerated	benign	deleterious
aHUS4	CFH	G>G/A	1	196646650	59	191	V158I,	0.000132	tolerated	benign	neutral
aHUS4	CFH	G>G/C	1	196711127	44	369	A1027P	0.00004119	tolerated	benign	neutral
aHUS1	CFB	A>A/C	6	31915584	50	567	I242L	0.00002559	tolerated	benign	neutral
aHUS3	CFHR3	G>G/T	1	196749023	55	190	G117V	N/A	deleterious	probably damaging	deleterious
aHUS5	THBD	C>C/T	20	23030015	59	87	A43T	0.00343	tolerated	benign	neutral
aHUS10	CFH	G>G/C	1	196712669	49	508	R1074P	0.00001647	deleterious	probably damaging	deleterious
aHUS10	CD46	CAG>C	1	207940331	25*	8*	S274YFS*11	N/A	N/A	N/A	N/A
aHUS6	CD46	G>G/C	1	207934766	50	701	W216C	N/A	deleterious	probably damaging	deleterious
aHUS8	CFH	G>G/T	1	196712682	51	332	R1078S	0.00005765	tolerated	benign	neutral
aHUS7	CFHR5	C>C/G	1	196973817	47	220	P453A	0.000008238	deleterious	probably damaging	deleterious

* This variant did not meet our filtering criteria, however has been previously reported (39) and verified via CLIA laboratory testing. Chr = chromosome, VAF = variant allele frequency, MAF = minor allele frequency as reported in the ExAC database.


% Cell Killing
100
80
60
40
20
0
HELLP Syndrome
Partial HELLP Syndrome
Atypical Hemolytic Uremic Syndrome
Thrombotic Thrombocytopenia Purpura
Healthy Pregnant Controls
Participant Groups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V158I
H371N
A1027P
R1074P
R1078S
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
1231 aa
CFHR1
L322Rfs*50
1 2 3 4 5
330 aa
del(CFHR1) homozygous
CFHR3
C15Y
G117V
1 2 3 4
330 aa
del(CFHR3-CFHR1) homozygous
CFHR5
S78P
Y279N
P453A
K562Nfs*24
1 2 3 4 5 6 7
569 aa
CFI
I416L
SRCR
LDL
LDL
S-S
Serine Protease
583 aa
C3
G542D
P890H
D1457H
MG MG MG MG MG MG LNK ANA MG MG CUB TED CUB MG NTR
1663 aa
CFB
I242L
1 2 3
VWA
Serine Protease
764 aa
CD46
W216C
S274Yfs*11
1 2 3 4
TM
CY
392 aa
Legend
aHUS
HELLP
partial HELLP
pregnant control

STROBE Statement—Checklist of items that should be included in reports of ***cohort studies***

	Item No	Recommendation
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found
Introduction		
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported
Objectives	3	State specific objectives, including any prespecified hypotheses
Methods		
Study design	4	Present key elements of study design early in the paper
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection
Participants	6	(*a*) Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up
		(*b*) For matched studies, give matching criteria and number of exposed and unexposed
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group
Bias	9	Describe any efforts to address potential sources of bias
Study size	10	Explain how the study size was arrived at
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding
		(*b*) Describe any methods used to examine subgroups and interactions
		(*c*) Explain how missing data were addressed
		(*d*) If applicable, explain how loss to follow-up was addressed
		(*e*) Describe any sensitivity analyses
Results		
Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed
		(b) Give reasons for non-participation at each stage
		(c) Consider use of a flow diagram
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders
		(b) Indicate number of participants with missing data for each variable of interest
		(c) Summarise follow-up time (eg, average and total amount)
Outcome data	15*	Report numbers of outcome events or summary measures over time
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included

		(*b*) Report category boundaries when continuous variables were categorized
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses
Discussion		
Key results	18	Summarise key results with reference to study objectives
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence
Generalisability	21	Discuss the generalisability (external validity) of the study results
Other information		
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based

*Give information separately for exposed and unexposed groups.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at http://www.strobe-statement.org.